Differential Regulation of mTORC1 Signaling in Brain Cells: a Novel Role for Astrocytic 4E-BP1

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1 Abbreviations

4E-BP	eukaryotic translation initiation factor 4E binding protein
AMPK	AMP activated protein kinase
Ax	anoxia
CNS	central nervous system
DIV	days *in vitro*
eIF2α	eukaryotic translation initiation factor 2 subunit α
eIF4E	eukaryotic translation initiation factor 4E
eIF4F	eukaryotic translation initiation factor 4F
eIF4G	eukaryotic translation initiation factor 4G
GSK3	glycogen synthase kinase 3
HIF	hypoxia-inducible factors
HRE	hypoxia response elements
Hx	hypoxia
IGF1	insulin growth factor 1
IRS1	insulin receptor substrate 1
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
Nx	normoxia
PDK	phosphoinositide-dependent kinase
PI3K	phosphatidylinositol 3- kinase
PIP2	phosphatidylinositol-4,5-phosphate
PIP3	phosphatidylinositol-3,4,5- phosphate
PTEN	phosphatase and tensin homolog

pVHL	von Hippel-Lindau tumor suppressor
Raptor	regulatory-associated protein of mTOR
REDD1	regulated in development and DNA damage response 1
Rheb	Ras homologue enriched in brain
Rictor	rapamycin insensitive component of mTOR
TOP	terminal oligopyrimidine
TSC	tuberous sclerosis complex

2 Summary

Neurons are fundamental for brain activity and tightly collaborate with astrocytes to guarantee proper functions of the CNS. However, they show extremely different sensitivity towards hypoxia, a deleterious factor of many neuropathologies such as stroke, cancer and Alzheimer's disease. While hypoxic insult triggers neuronal damage, sustained oxygen deprivation can activate astrocytic cells, promoting their proliferation and inducing gliosis, mechanisms that to a large extent can lead to brain injury. During oxygen deprivation most cells undergo decreased protein synthesis via mTORC1 inhibition to reduce energy consumption and modulate transcription regulation via HIF-1. The mTORC1 pathway controls the synthesis of the majority of proteins and ribosomal subunits by regulating cap- and TOP-dependent mRNA translation. This cascade has been extensively studied in cancer cells, however recently unbalanced mTORC1 signaling was also implicated in neurological diseases. The main focus of this study was to elucidate the role of mTORC1 pathway in astrocyte and neuronal response to oxygen deprivation. In particular, we were interested in understanding how mTORC1 signaling modulates overall protein synthesis and energy levels in brain cells with different sensitivity to hypoxia. In neurons mTORC1 activity is partially inhibited already at baseline (normoxic) conditions and inhibition of cap-dependent translation does not affect the overall protein synthesis rate. On the other hand, astrocytic mTORC1 pathway is insensitive to low oxygen levels but is completely inhibited during ischemia (oxygen and glucose deprivation). Although ischemic dephosphorylation of 4E-BP1 prevents the interaction of eIF4E with eIF4G and impedes cap-dependent

translation in astrocytes, it has no added inhibitory effect on global protein synthesis rate and results in a marked fall of cellular ATP. Overall pharmacological inhibition of mTORC1 via rapamycin treatment does not alter global protein synthesis regulation or ATP levels in either astrocytes or neurons, but does slow HIF-1α accumulation in either cell type.

In direct relation to this we showed in astrocytes that knockdown of 4E-BP1 during normoxia, when the protein is hyper-phosphorylated by mTORC1, reduced protein synthesis rate and cell proliferation, while during oxygen deprivation did not affect either mRNA translation or cell cycle.

Taken together the present work reveals that neurons and astrocytes differentially inhibit mTORC1 signaling during stress conditions and that this signaling cascade is not implicated in overall protein synthesis and ATP levels regulation in either cell type. Moreover, in astrocytes the mTORC1 effector 4E-BP1 not only acts as brake of cap-dependent translation during ischemia but also as stimulator of cell growth and proliferation in normoxic conditions.

3 Zusammenfassung

Nervenzellen sind die Grundlage jeglicher Hirnfunktion. Ihre Zusammenarbeit mit den Hauptkomponenten der Glia, Astrozyten, stellt sicher dass die Aktivitäten des ZNS im physiologischen Rahmen bleiben und im engen Einklang mit der Umwelt stehen. Allerdings zeigen beiden Zelltypen eine stark unterschiedliche Sensitivität gegenüber Hypoxie (Mangelversorgung an Sauerstoff), einem Faktor also, der die Pathogenese bei vielen Erkrankungen des Nervensystems wie Schlaganfall, Hirntumoren und Alzheimer Demenz antreibt und die Prognose verschlechtert. Während andauernde oder strenge Hypoxie zu irreversiblen neuronalen Defiziten durch Zelltod führt, werden Astrozyten durch dieses Signal und die absterbenden benachbarten Neurone zu hypertrophem, bisweilen auch hyperplastischem, Wachstum angeregt. Mit dieser reaktiven Astrogliose geht eine Vernarbung des betroffenen Hirnareals einher, die Zell-Zell-Kommunikationen und Signalausbreitung massiv beeinträchtigen kann, gleichermaßen aber auch eine Abgrenzung des geschädigten Gewebes bedeutet (Barrikadenfunktion). Die meisten Zellen reagieren auf starke Gewebshypoxie mit der Hemmung des mTORC1 Kinase Komplexes und, infolge dessen, einer verlangsamten Proteinsyntheserate. Diese Maßnahme soll den Energieverbrauch in Zeiten mangelhafter ATP Produktion reduzieren, sowie vielfache Genexpressionen durch den Transkriptionsfaktor HIF-1 modulieren. Es ist bekannt dass der mTORC1 Signalweg, je nach Umweltsignal (z.B. O_2 Normal- oder Mangelversorgung), die Synthese der Mehrheit an Zellproteinen und von ribosomalen Untereinheiten durch Abstimmung der Translationsrate von cap- bzw. TOP-abhängigen mRNAs koordiniert. Bislang wurde die Kaskade vor allem

in Krebszellen untersucht, kann jedoch auch in Neuropathien dereguliert vorliegen.

Das zentrale Anliegen dieser Doktorarbeit war es die Rolle des mTORC1 Signalweges in der astrozytären und neuronalen Reaktion auf eine Mangelversorgung an Sauerstoff (inkl. Mangelversorgung: Hypoxie; ausbleibende Versorgung: Anoxie) bzw. einen unterbrochenen Zufluss an biochemischen Substraten (z.B. Glukose) und Sauerstoff (Ischämie) zu untersuchen. Dabei wollten wir vor allem verstehen wie sich die mTORC1 Kaskade unter diesen verschiedenen Stressbedingungen auf die globale (i.e. Translationstypus übergreifende) Proteinsynthese und ATP Fließgleichgewichte in Hypoxie sensitiven (Neuronen) und toleranten (Astrozyten) Hirnzellen auswirkt.

Wir fanden dass der neuronale mTORC1 Signalweg bereits unter Basisbedingungen (Normoxie) partiell gehemmt vorlag, möglicherweise in Korrelation mit dem post-mitotischen Zustand von Nervenzellen des Säugerhirns. Dementsprechend war die Stress-induzierte (Hypoxie, Anoxie) Steigerung der Hemmung von cap-abhängiger Translation moderat und hatte keine Auswirkungen auf die globale Proteinsynthese. Die Gliazell-Kaskade, vor allem von mTORC1 zu Effektor 4E-BP1, war hingegen in normoxischen Zellen maximal aktiv, jedoch refraktär gegenüber Hypoxie oder Anoxie, und wurde erst durch Ischämie komplett inaktiviert. Dieser Totalverlust von mTORC1 Aktivität in ischämischen Astrozyten bedingte eine quantitative Dephosphorylierung von 4E-BP1 und maximale kompetitive Interferenz mit der für die Translation unerlässlichen eIF4E-eIF4G Interaktion. Aber, selbst diese strikt gehemmte cap-Translation korrelierte auch in Gliazellen nicht mit einer maximal verlangsamten

globalen Proteinsyntheserate, weshalb ein massiver Schwund an ATP in ischämischen Gliazellen zu beobachten war. Desgleichen beeinflusste auch die pharmakologische Inaktivierung von mTORC1 durch Gabe von Rapamyzin (Sirolimus), über das jeweilige hypoxische, anoxische oder ischämische Niveau hinaus, weder die globale Proteinsynthese noch das ATP Fließgleichgewicht von Glia- und Nervenzellen. Allerdings wurde die Akkumulation von HIF-1α infolge dieser Stresssignale durch Rapamyzin in beiden Hirnzelltypen deutlich verringert.

Zu guter Letzt untermauerte diese Arbeit dass Vorhandensein einer neuartigen, weniger gut verstandenen Funktion von 4E-BP1 in oxygenierten Astrozyten. Hier wird der Faktor durch aktiven mTORC1 Komplex hyper-phosphoryliert. Der siRNA-bedingte knockdown von 4E-BP1 ging nur in normoxischen Gliazellen (i.e. Funktionsverlust von hyper-phosphoryliertem 4E-BP1), nicht aber anoxischen oder ischämischen Pendants, mit einer Reduktion von Proteinsynthese- und Zellproliferationsrate einher.

Zusammenfassend erarbeitete diese Studie wesentliche Aspekte zu der unterschiedlichen Stress Reaktion des mTORC1 Signalweges in Hypoxie-sensitiven Neuronen und Hypoxie-toleranten Astrozyten. Allerdings hat dieser Signalweg in beiden Zelltypen keinen merklichen Einfluss auf die Regulation der globalen Proteinsynthese und des Energiehaushaltes. Die pharmakologische Hemmung des mTORC1 Signalweges bedingt hingegen auch in ZNS Zellen ein verändertes HIF-abhängiges Stress Transkriptom. Bezüglich der Translationskontrolle durch mTORC1-4E-BP1 ist anzunehmen dass die Produktion einiger weniger, für das Zellüberleben wesentlicher, mRNAs durch diesen Signalweg reguliert wird. Um welche Transkripte es sich dabei handelt

müssen künftige Studien ergeben. Schließlich konnte für primäre Astrozyten gezeigt werden das 4E-BP1 eine Ying-Yang-artige Doppelfunktion innewohnt: eIF4E-bindende Translationsbremse im dephosporylierten Zustand, also unter ischämischen Kinase-hemmenden Bedingungen, und Synthese-/Wachstum-fördernder Faktor sobald die Zellen den Stress überwunden haben und wieder mit Sauerstoff und Energiesubstraten versorgt werden.

4 Introduction

4.1 Brain physiology

The brain represents the fundamental link between organism and environmental stimuli, generating patterns of muscle activity and driving secretion of molecules called hormones, it carries out disparate functions such as behavior control, comprehension, memory, motor commands, signals interpretation and decoding. Brain is highly dependent on the supply of nutrients and oxygen, and it requires a correct homeostasis to ensure coordination of both cognitive and non-cognitive processes. Impaired developmental mechanisms as well as external factors can cause structural, biochemical or electrical alterations and negatively influence normal brain activity. Although it constitutes only 2-3% of the total body weight, the brain utilizes approximately 20% of the body oxygen consumption (1). Decreased oxygen delivery, as a result of impaired blood flow, cardiovascular and/or respiratory disease as well as hemorrhage, can lead to critical oxygen tension (in adult mammals is between 25 and 40 mmHg) and cause up to 90% decline of energy in as little as 5 min (2). Since oxygen metabolism provides energy for normal functions of the central nervous system (CNS), limited oxygen supply is an important and frequent pathological component of many brain diseases and pathologies such as stroke, cancer and Alzheimer's disease (3).

Of the many different cell types found in the brain (e.g. neurons, astrocytes, pericytes, endothelial cells) that contribute to the correct activity of the CNS, neurons and astrocytes seem to play major roles, thus different in both physiological and pathological events.

4.1.1 Neurons and stress sensitivity

Neuronal cells are fundamental for brain activity. They are electrically excitable cells that process and transmit information via electrical and chemical signals to the whole body. Although various types of neurons are characterized by different morphology, they all consist of four regions: 1) dendrites, the projections receiving electrochemical stimulation from other neural cells, 2) cell body (soma), containing nucleus and cytoplasm, 3) axon, the output fiber conducing electric impulses and 4) axon terminals, the projections transmitting signals to the other cells (Fig. 1, micrograph of cultured murine 6 DIV neurons that have been used in this project).

Communication between neurons and other cells takes place at specialized sites called synapses, where the information flows from one cell to another by release of molecules known as neurotransmitters. Once the signal reaches the dendrites, the propagation of the impulse occurs in only one direction and involves the opening and closing of ATP-driven ion channels along the axon. Action potentials and excitatory postsynaptic currents generated by ion fluxes through the channels represent the major energy expenditure for the brain (1) and are fundamental for the correct transmission of signals.

Maintenance of functional neurons is essential through life to guarantee normal brain activity. Disturbance of brain homeostasis due to alteration of different factors, e.g. oxygen supply and energy metabolism, not only affects the brain but can influence the whole body. Memory, learning, locomotion and senses are only few of the aspects that can be altered by neuronal loss during CNS injury (4–6). Neurons use most of the energy consumed by the brain to generate action potentials and restore ionic concentrations after synaptic transmission (7).

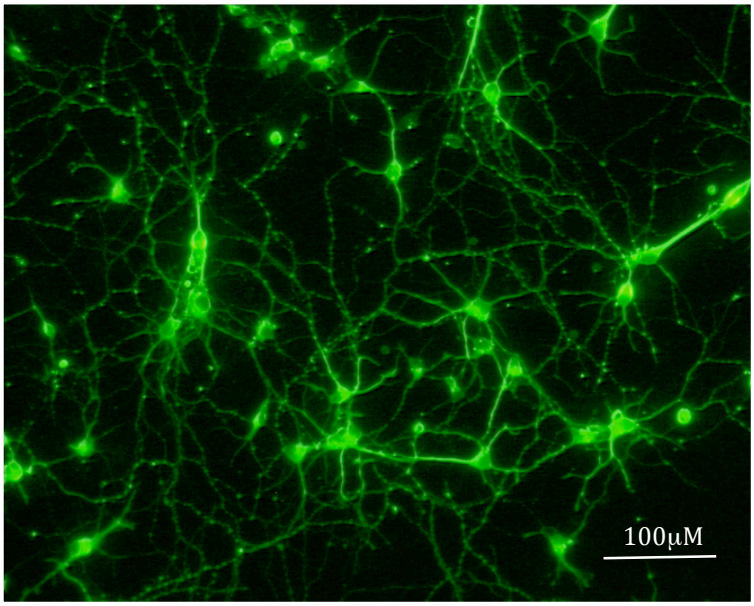

Fig. 1 Neurons in culture. Micrograph of primary murine neurons (6 DIV) that have been used in this project. Cells were stained with TUJ-1 (neuronal-specific tubulin). Picture provided by Dr. O. Ogunshola.

A drop in cerebral perfusion, hypoxia, hypoglycemia, and severe anemia can cause a critical energy decrease and trigger neuronal damage. Indeed, neurons are considered to be the most sensitive cells of the CNS and there is large evidence that oxygen deprivation can induce neuronal cell injury, neurodegeneration and cell death within minutes (8). Mammalian neurons exposed to acute hypoxia undergo neurophysiological changes as a consequence of loss of ionic homeostasis and abnormal release of excitatory neurotransmitters (9). The early neuronal response to oxygen deprivation involves inhibition of the electrochemical gradient regulator Na^+-K^+-ATPase, cellular edema and subsequent neurodegeneration (10). Moreover, energy fall may induce glycolysis (11), increase free radical formation and cytosolic calcium levels (12), leading to cell death. Indeed, in a neonatal model of hypoxic-ischemic injury apoptotic and necrotic neuronal death contributed to brain damage (13). Moreover, acute exposure to high altitude, where reduced air pressure creates low partial pressure of oxygen, has been shown to lead neuronal loss, alter neuro-transmitter metabolism and cellular energy crisis in old rats (14).

Although it is known that hypoxia characterizes many neurological diseases, such as stroke, Alzheimer's and Parkinson's diseases (15, 16), the pathways contributing to hypoxic-driven neurodegeneration are still not fully elucidated. Notably, neurons alone cannot perform all sophisticated brain functions, therefore other cells (e.g. astrocytes, oligodendrocytes, pericytes, endothelial cells) support and cooperate with them to allow proper CNS activities. In the complex network of cellular brain connections, astrocytes play a significant role in maintaining the neuronal environment and providing nutrients (Fig. 2,

schematic representation of substances released from astrocytes at the synapsis to provide local metabolic support to neurons).

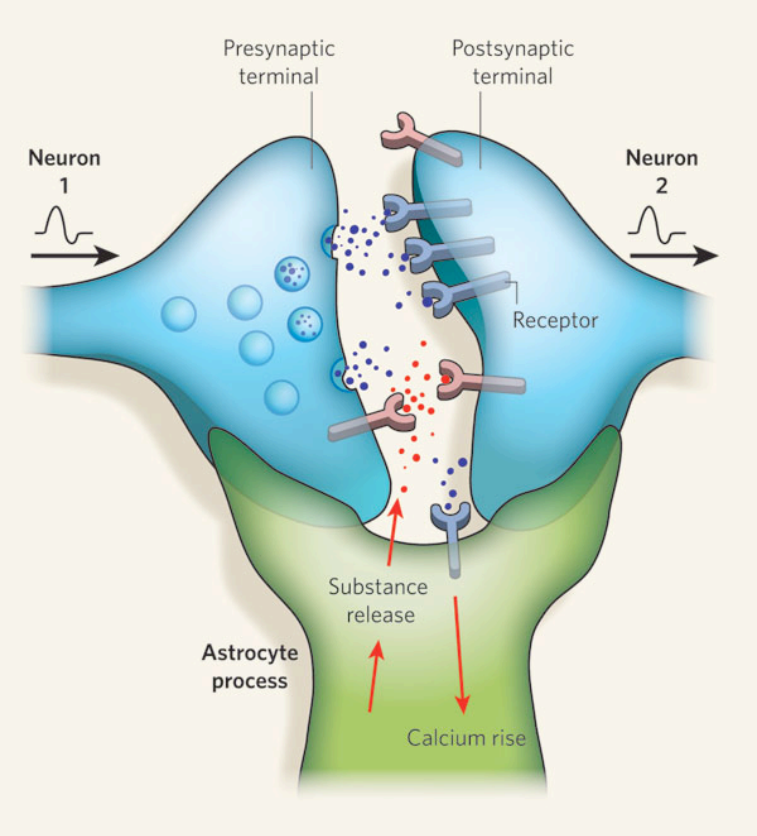

Fig. 2 Schematic representation of an astrocyte interacting with neurons at the synapse. At the synapse, astrocytes interact with neurons through their processes. Astrocytic and neuronal cells both express many common receptors. Therefore release of neurotransmitters from the neuronal presynaptic terminal also stimulates astrocytes. Activation of astrocytic receptors triggers the increase of intracellular calcium and, in turn, the release of active substances, which provide local metabolic support to neuronal cells and participate to synaptic transmission. Figure taken from (17).

4.1.2 Astrocytes and their responses to CNS injury

Astrocytes are the most abundant cells in the mammalian brain (Fig. 3, micrograph of primary rat astrocytic cultures that have been used in this project). The proportion of astrocytes to neuronal cells seems to increase dramatically during the course of evolution, ranging from a 1:6 ratio in *Caenorabditis elegans*, where neurons exceed astrocytes, to a 3:1 ratio in the cortex of lower mammals. Astrocytes are specialized glia cells performing a broad number of functions: maintenance of the homeostasis, metabolic and anti-oxidant support and neuroprotection (18). Therefore they not only contribute to normal performance of the healthy CNS, but also respond to all forms of brain injury. Astrocytes are essential for synaptic transmission (18) and provide local metabolic support to neurons. Being the exclusive holder of glycogen energy stores in the brain, they are fundamental for neuronal survival and function (19) even during hypoglycemia and during periods of high neuronal activity. Indeed, glycogen breakdown leads to the production of lactate, which is then exported to neurons as a major energy source, thus conferring to astrocytes the critical role in supporting neuronal metabolism by the "lactate shuttle" (19). Additionally, astrocytes show high resistance to oxidative stress due to their elevated levels of antioxidants such as glutathione and the ability to overexpress reactive oxygen species (ROS) scavengers metallothionein and superoxide dismutase (SOD) enzymes during cerebral ischemia (20, 21).

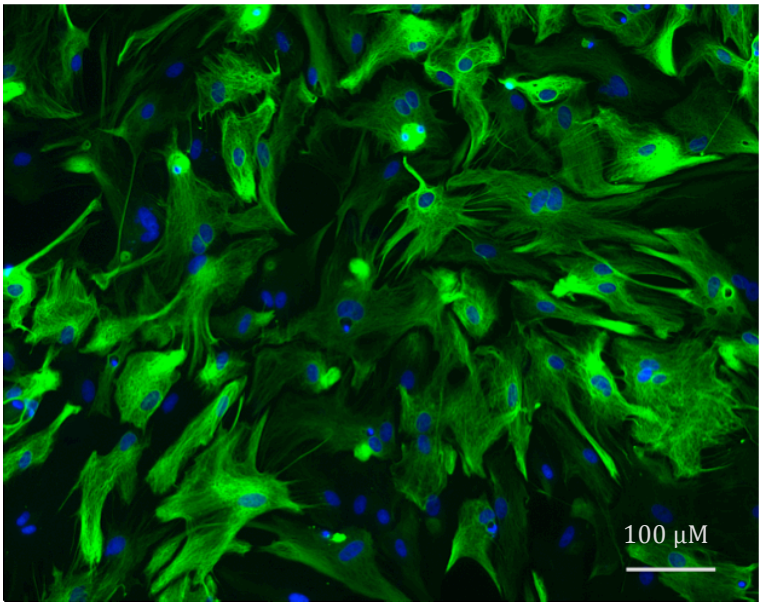

Fig. 3 Astrocytes in culture. Micrograph of primary rat astrocytic cultures that have been used in this project. Cells were stained with GFAP (glial acidic protein; green) and DAPI (blue). Picture provided by S. Engelhardt.

Notably, astrocytes can withstand extreme insults for a prolonged period of time (22, 23). During oxygen/glucose deprivation these cells are able to switch from an aerobic to anaerobic metabolism (24, 25), contribute to injury response mechanisms and preserve the host tissue integrity (26). Astrocytes are also considered the main source of erythropoietin (EPO) in the brain (27, 28), which protects neurons from ischemic insults *in vitro* (29, 30) and in *in vivo* stroke models (31–33). Moreover, astrocytes can induce and secrete vascular endothelial growth factor (VEGF) (23, 34), activating angiogenic and neuroprotective mechanisms (35). However during stress or injury astrocytes may become highly activated and undergo gliosis, a process involving proliferation, hypertrophy of cellular processes and altered expression of many proteins (36), that to large extent can be less desirable and perpetuate damage (37). Therefore, following CNS injury astrocytic response can include changes in gene expression, hypertrophy and cell growth (38, 39). Particularly, hypoxic and ischemic insults can activate and promote proliferation of resident astrocytes both *in vitro* (23), and *in vivo* (26, 40). Although gliosis is believed to provide a permissive environment to preserve neuronal functions (41), hyperproliferation and formation of a glial scar may also be detrimental and impede axon regeneration (42, 43).

To date despite being clear that astrocytes play a fundamental role in supporting normal brain functions and maintaining CNS homeostasis during insults, molecular changes during O_2 deprivation are only poorly understood.

4.2 Physiological response to hypoxia

O_2 is fundamental for life since it is part of proteins, nucleic acids, carbohydrates, fats and water, the main constituents of cells. Moreover, O_2 is essential to synthetize the ATP that is used as a fuel to perform many activities. The ability to maintain O_2 homeostasis is essential for the development, growth and survival of multicellular organisms. Thanks to the evolution of complex structures such as respiratory and cardiovascular systems, O_2 is properly distributed from organs to tissues to cells and functions as the terminal electron acceptor during mitochondrial oxidative phosphorylation ensuring ATP production. A switch from aerobic to anaerobic metabolism during decreased O_2 supply reduces energy levels (44) and challenges organisms, although tolerance to O_2 depletion varies considerably among species (45). Sensitivity to O_2 availability is also markedly different among organs in the same organism. Inside the human body normal O_2 levels are considerably lower than the 20.9% O_2 we breathe, e. g. in lung parenchyma, liver, kidneys and heart O_2 varies from 14 to 4%, while in the eye from 1 to 5% and in the brain from 0.5 to 7% (46).

Hypoxia is best defined as the condition where oxygen partial pressures (pO_2) have fallen to, or below, a tissue (cell)-specific critical value that separates oxidative from anaerobe metabolism (47). Tissue hypoxia can occur during both physiological and pathological events. In the human body different processes are activated to allow acclimatization to the hypobaric hypoxia at high altitude, where hypoxic conditions may occur (48). Physiological adaptations such as increased ventilation, cardiac output, vascularization of tissues and cellular modifications aim to maintain the oxygen carrying capacity of the system during periods of reduced pO_2 and ensure better utilization of the available O_2 (49).

Other mechanisms such as development of the mammalian embryo (50), control of stem cell differentiation and angiogenesis (46, 51) require reduced O_2 supply, therefore indicating O_2 deprivation as regulatory mechanism during normal conditions.

4.2.1 Pathophysiological response to hypoxia

Disturbed O_2 homeostasis is a typical feature of many pathological conditions that often exacerbates disease progression. Impaired blood flow, as observed during myocardial and cerebral ischemia and other diseases, can cause significant brain hypoxia (49). In these injuries detrimental outcome occurs not only as a direct consequence of hypoxic conditions but also due to the burst of reactive oxygen species (ROS) following the restoration of the blood flow (49). Moreover, periods of chronic hypoxia can predispose to development of neurodegeneration. Increased risk of Alzheimer's disease has been associated with hypoxia arising from cardiovascular and respiratory disorders (15, 52) and impaired oxygen supply can also contribute to amyotrophic lateral sclerosis (ALS) (53) probably due to motor neuronal death (54). Solid tumors are characterized by regions of low oxygen tension that activate complex pathway cascades for the formation of new blood vessels, a pathological mechanism called neoangiogenesis (55), to increase O_2 and nutrients supply and allow tumors growth and survival (56).

Although the involvement of reduced oxygen availability was demonstrated in both physiological and pathological mechanisms, the molecular pathways involved still need to be fully elucidated. Low oxygen levels cause modulation of

transcriptional responses, particularly via the hypoxia-inducible factors (HIF), but cellular adaptation is primarily affected by regulation of translational mechanisms.

4.2.2 HIF-mediated transcriptional regulation of hypoxic responses

Hypoxia-inducible factors (HIF) are mainly responsible for cellular adaptation to oxygen deprivation. HIF-1 was identified about two decades ago by Semenza and Wang (57, 58) and since then different isoforms have been discovered. HIF-1 plays and essential role during oxygen homeostasis by inducing more than 70 validated, and perhaps several hundred of potential, hypoxia-responsive gene targets in all mammalian cell types examined (59, 60). Cellular changes induced by HIF-1 affect glucose transport, glycolytic versus mitochondrial activities, survival and proliferation (61), and ensure systemic oxygen homeostasis by increasing EPO, angiogenesis and dilation of blood vessels (62).

HIF is a heterodimer composed of an oxygen-regulated HIF-α subunit and a constitutively expressed HIF-β subunit (also known as aryl hydrocarbon receptor nuclear translocator or ARNT). Alpha and beta subunits of HIF are members of the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain family of transcription factors that mediate their dimerization via the HLH/PAS motifs and the binding to DNA (63). To date, three HIF-α (HIF-1α, HIF-2α and HIF-3α) and HIF-β (ARNT, ARNT2 and ARNT3) isoforms have been described among which HIF-1α and HIF-2α are the best characterized. Although oxygen-independent mechanisms can also promote HIF-α stabilization via RACK1 or GSK3β (64), modulation of HIF-α abundance mainly depends on O_2 availability

and occurs at many levels: mRNA expression, protein stability, nuclear translocation and transcriptional activity. At the protein level, regulation of HIF-α subunits is mediated by hydroxylation of prolyl residues, followed by the von Hippel-Lindau tumor suppressor (pVHL)-dependent ubiquitylation and proteasomal degradation (Fig. 4). During normoxia, or in re-oxygenating cells, human HIF-1α is hydroxylated at two conserved proline residues (Pro402 and Pro564) by prolyl hydroxylase domain proteins (PHD1, 2 and 3). A second O_2-requiring hydroxylation by the asparaginyl hydroxylase FIH-1 (factor inhibiting HIF-1) modifies an asparagine within the C-terminal transactivation domain of HIF-1α or -2α, thus preventing recruitment of the coactivator proteins p300/CBP, and transactivation of target genes under high oxygen partial pressure (65). PHD and FIH-1 activity decreases during hypoxia, which allows the rapid accumulation of transcriptionally active HIF-1α/-2α isoforms. Thereafter the factors translocate to the nucleus where they dimerize with HIF-β/ARNT partner proteins. HIF-1 and HIF-2 heterodimers work as transactivators i.e. they induce expression of both shared and specific target genes involved in important processes such as metabolism and cell growth by binding to hypoxia response elements (HREs).

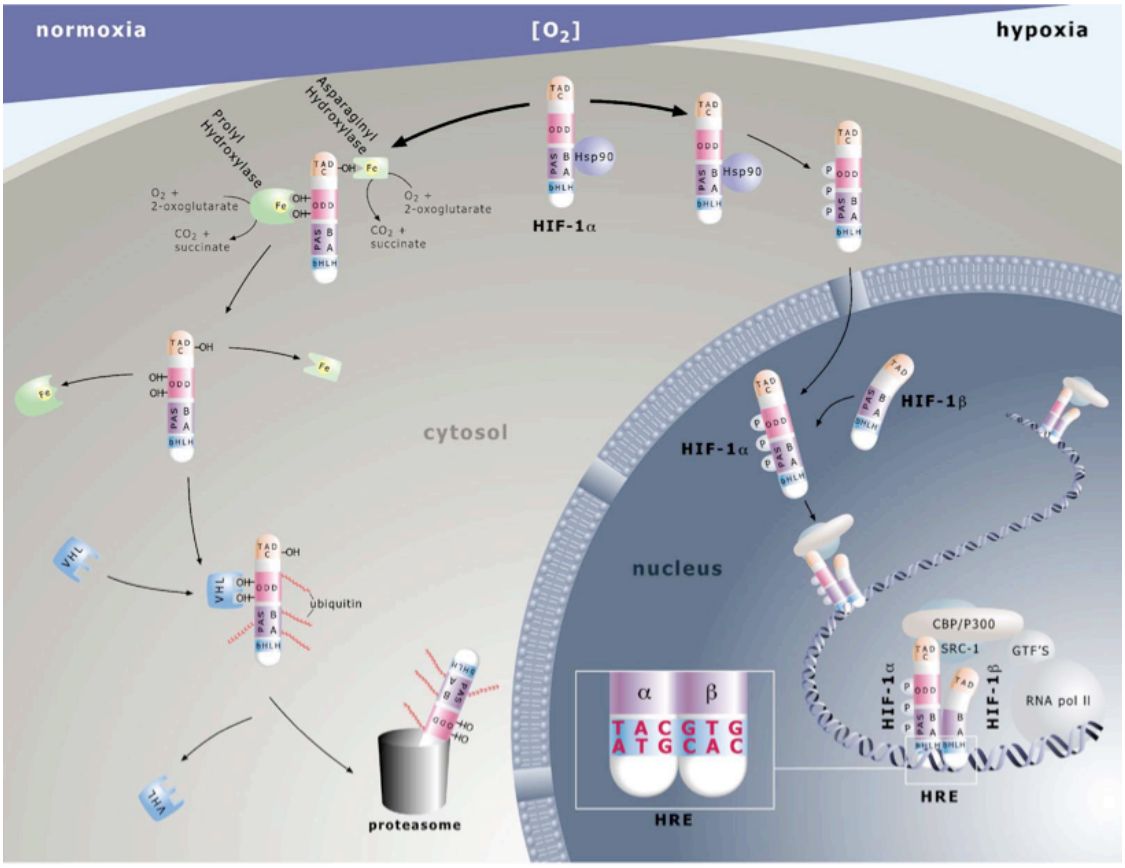

Fig. 4 HIF-1α regulation by prolyl-hydroxylation. a) In the presence of O_2 (normoxia), Fe^{2+}, 2-oxoglutarate (2-OG) and ascorbate HIF-α is hydroxylated by prolyl hydroxylase domain proteins (PHD1, 2 and 3). Poly-hydroxylated HIF-1α binds to von Hippel-Lindau tumor suppressor (pVHL), is poly-ubiquitylated and therefore degraded by 26S proteasome complex. Acetylation of HIF-1α (OAc) also promotes pVHL binding and following degradation. b) During hypoxia, hydroxylation is inhibited, HIF-1α is no longer degraded but translocates to the nucleus where it dimerises with HIF-1β. HIF-1 complex recruits the co-activators p300/CBP and SRC-1, binds the hypoxia-response elements (HREs) of the target genes and modulates the activity of the general transcription factor (GTF)/RNA polymerase II machinery in a hypoxia-responsive way. Targets genes regulate many different functions. Figure from (66).

Due to the broad range of cellular proteins they activate, it is not surprising that HIF-1/-2 are required during physiological processes, such as embryonic survival and development of the circulatory system (67–69). Messenger RNAs of HIF-1α and -2α are constitutively expressed in cultured cells and the normal adult brain. However, focal cerebral ischemia is known to dramatically induce HIF-1α mRNA expression in peri-infarct areas (70). It has been suggested that increased HIF-1 activity could be beneficial for Parkinson's and Huntington's diseases, although the mechanisms are still not clear (71). However, the role of HIF-1 during hypoxia can have either favorable or detrimental effects. While, on the one hand, several studies have confirmed a pro-survival role of HIF-1 (72, 73), it has also been shown that in neuronal model of ischemic injury HIF can promote cell death (74).

Even though much is known about HIF-1α degradation, specific upstream mechanisms able to impinge on the steady state of the subunit still need to be fully elucidated. Of particular interest for this study is the widely held notion that mammalian target of rapamycin (mTOR) can activate HIF-1 (75). It was recently shown *in vivo* that expression of HIF-1α and its target vascular endothelial growth factor (VEGF) is reduced upon mTOR inhibition in developing brain (76), thus supporting the hypothesis of such pathway being involved in the regulatory mechanism of HIF-1. Moreover, in non-excitable cells mRNA translation uses more cellular energy than any other biosynthetic activity (77) and accordingly translational rate in such cells is extremely sensitive to O_2 availability and cell metabolism and is finely regulated at many levels.

4.2.3 mRNA translation

Gene expression is a complex process regulated by many different and finely tuned mechanisms that counteract changes in the microenvironment and trigger cellular responses to stimuli. Regulating the content and level of proteins in cells, gene expression drives differentiation, proliferation, apoptosis, transformation and many other cellular mechanisms. It is, therefore, not surprising that protein expression is finely controlled at many levels. Transcription of genes into mRNAs exerts the primary control on protein regulation occuring in the nucleus of cells and depends on DNA sequences (e.g. promoters and enhancers) and modification (e.g. methylation), as well as activity of transcriptional regulatory proteins (e. g. HIF; see section 4.2.2) (78). However, it was recently shown that the rate of mRNA translation is intensively regulated during cell differentiation (79) and is decreased under stress conditions as first line of defense mechanism (see section 4.2.4), therefore underlying the importance of the protein synthesis modulation in response to different stimuli. Translation of mRNAs into proteins occurs in the cell cytoplasm and, is tightly dependent on post-transcriptional modification of the mRNA, such as addition of the 5' cap (see section 4.3.3.1) and the poly (A) tail structures (80), and the recruitment of protein factors devoted to the assembly of the translation machinery (see section 4.3.3.1). Coordinated recruitment of translational factors to the mRNA is determined by the activity of different kinases and the modulation of signaling cascades via diverse stimuli (81).

4.2.4 Translational regulation during hypoxia

Due to the strong dependence of ongoing mRNA translation on abundant O_2 delivery to the cells, regulation of protein synthesis is important for rapid response to hypoxic conditions. Hypoxia modulates cellular protein synthesis at least at two levels: i) through the inhibition of the overall level of the mRNA translation and ii) through differential protein expression (82). Decrease of the overall protein synthesis during O_2 deprivation requires inhibition of ribosomal clustering and mRNA initiation (82). It was shown that within minutes of anoxia HeLa cells reduce the number of ribosomes per translated transcript (83). Similarly, loss of polysomes has been described to occur in different types of deoxygenated cells such as human cancer cell lines as well as primary prostate adenocarcinoma and immortalized human fibroblasts (82). These findings suggest the reversible arrest of protein synthesis to represent a first line of defense mechanism that is common among primary and transformed cells subjected to acute O_2 deprivation. Translational control of constituents of the ribosomal machinery is mainly conferred via the so-called 5' terminal oligopyrimidine tract (TOP) seen in a small number of mRNAs (84) while mRNAs encoding the majority of cellular proteins are regulated by a different path, the so-called 5'-cap-dependent mRNA translation (see section 4.3.3). Cap-dependent translation is modulated by a complex series of phosphorylation events in response to diverse environmental stimuli, a mechanism where the mTORC1 complex seems to play a central role (85).

Production of selective "survival" transcripts and polypeptides evidently requires changes in specific transcription and translation processes. Indeed, selection of gene expression during O_2 deprivation does not only depend on

transcriptional regulation, mediated by HIF and other transcription factors, but is it also influenced at the translational level (86). While life-threatening challenges may yield a marked slow-down of global protein synthesis rate, translation of some survival factors, such as VEGF and HIF-1α itself, is maintained, if not activated (87, 88). Up to 5% of cellular mRNAs are believed to be translated via a cap-independent initiation mechanism, for example by internal ribosome entry sites (IRES) (89). In such mechanisms, ribosomes will be directly recruited to mRNA to internal entry site elements, therefore bypassing the inhibition of the bulk of the (cap-dependent) translation initiation machinery.

4.3 mTOR kinase

The target of rapamycin (TOR) kinase was originally isolated in budding yeast *Saccharomyces cerevisiae* (90) as target of the antifungal macrocyclic lactone antibiotic rapamycin (aka sirolimus), through identification of mutants resistant to the drug. Shortly after this discovery, the mammalian TOR (mTOR) homolog was characterized by different groups (91, 92). Since then mTOR´s impact on many physiological and pathological mechanisms became more and more evident (93, 94).

mTOR, also known as FRAP (FKBP12-rapamcyin-associated protein), RAFT1 (rapamycin and FKBP12 target), RAPT 1 (rapamycin target 1), or SEP (sirolimus effector protein), is a serine/threonine kinase of 289 kDa (2549 amino acids) and member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family (93). Amino acid sequences of the mTOR protein of human, rat and mouse share

95% identity, while the human sequence of mTOR displays 42 and 45% identity with those of yeast TOR1 and TOR2, respectively (95). The importance of this kinase is clearly highlighted by its genetic conservation among species and is further confirmed by embryonic lethality in mice upon genetic mTOR loss-of-function (96). mTOR is believed to be the central regulator in coordinating cellular activities with the presence and abundance of many environmental factors such as nutrients, growth factors, energy and oxygen supply. Thus it plays a fundamental role in modulating cell growth, proliferation, survival and development in response to environmental changes. Dysfunction of mTOR signaling has been associated with different pathologies including metabolic disease, neurodegenerative disorders and cancer (97).

The mTOR kinase regulates cellular mechanisms within the context of two protein complexes commonly referred as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (98). Of note, mTORC1 is involved in the regulation of translational initiation and ribosome biogenesis, while mTORC2 regulates cytoskeletal organization.

4.3.1 mTOR complexes

Both mTORC1 and mTORC2 are structurally distinct multi-protein complexes, which phosphorylate different substrates and regulate distinct cellular cascades.

The mTORC1 is the master controller of energy consumption invested into protein synthesis, thus coordinates cell growth and proliferation as modulated by mRNA translation. It consists of mTOR kinase, G protein beta subunit-like (GβL; also known as mLST8), regulatory-associated protein of mTOR (raptor)

and proline-rich protein 40 (PRAS40). GβL, present in both mTORC1 and mTORC2 complexes, constitutively binds the catalytic domain of the mTOR kinase and enhances its activity (99). The subunit raptor functions as scaffold for recruiting substrates, therefore controls specificity of the complex and brings proteins such as ribosomal protein S6 kinase 70kDa (S6K) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) in the proximity of the kinase (100). The last partner of mTORC1, PRAS40, functions as direct inhibitor of the binding of the different proteins with the enzyme during nutrient deprivation (101, 102). The binding partners of mTORC2 are: mTOR, GβL, rapamycin insensitive component of mTOR (rictor) and the recently identified proteins SIN1 and proline-rich protein 5 (PRR5) (103, 104). Similar to raptor subunit in mTORC1, rictor is responsible to recruit substrate to the mTORC2 complex (105) while the last identified SIN1 and PRR5 have been shown to be important in maintaining mTORC2 stability and regulate its kinase activity (104, 106). However, beyond the modulation of mass accumulation and cell size via cytoskeletal organization by mTORC2, other functions and principles of the regulation of this complex are far less known (98).

Of the two mTOR complexes only mTORC1 is sensitive to the allosteric inhibition by rapamycin. This selective effect is due to the binding of the drug to the intracellular receptor FK506 binding protein 12 (FKBP12), which then directly interacts with the FKBP-rapamycin-binding (FRB) domain of mTOR (107). However, although mTORC2 is not inhibited by FKBP12/rapamycin complex, long-term drug exposure is known to reduce its activity (108) and this has been shown to be responsible for the side effects of rapamycin *in vivo* (109).

4.3.2 Upstream of mTORC1

The regulation of translational rate plays a fundamental role in many cellular activities; therefore a complex orchestra of proteins finely tunes this process at many levels (Fig. 5). As stated above, mTORC1 coordinates the initiation of the translational mechanism with the presence and abundance of stimuli such as: growth factors, nutrients, energy and stress signals.

4.3.2.1 Regulation of mTORC1 by growth factors and nutrients

Growth factors such as insulin initiate mTORC1 signaling via activation of the kinases phosphatidylinositol 3- kinase (PI3K) and AKT. The PI3K/AKT cascade relays signals coming from active receptor tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), platelet-derived growth factor receptor (PDGFR) via the insulin receptor substrate 1 (IRS1) (Fig. 5). One of the best characterized cascades is activated by insulin growth factor 1 (IGF1) (110). The binding of IGF1 or insulin to the IGFR triggers autophosphorylation of the receptor on different tyrosines and subsequent recruitment of IRS1 and PI3K, which is then activated (110). Next, PI3K catalyzes the conversion of phosphatidylinositol-4,5-phosphate (PIP2) to phosphatidylinositol-3,4,5- phosphate (PIP3) that allows the recruitment of phosphoinositide-dependent kinase 1 (PDK1) to the cell membrane, which then phosphorylates AKT at Thr308 (111). A second phosphorylation at the Ser473 triggered by mTORC2 complex fully activates AKT (112), which then phosphorylates the tuberous sclerosis complex 2 (TSC2) subunit of the TSC1/2 complex, blocking its inhibitory role and, in turn, activates mTORC1 via GTP-

bound Ras homologue enriched in brain (Rheb) (113). In its GTP-bound form, Rheb functions as indispensable activator of mTORC1 and TSC1/2 complex exerts its inhibitory role by converting Rheb into its GDP-bound inactive state (114). Although the mechanism is not completely elucidated, it is well accepted that GTP-bound Rheb activates mTORC1, hence allowing the phosphorylation of its effectors S6K1 and 4E-BP1.

The PI3K/AKT/mTORC1 pathway can be regulated at many levels. The best known antagonists of this cascade are the TSC1/TSC2 complex (previously described in this section), AMP-activated protein kinase (AMPK) (see section 4.3.2.2) and the phosphatase and tensin homolog (PTEN) phosphatase. PTEN acts upstream of the pathway and specifically catalyzes the dephosphorylation of PIP3 back to PIP2, thus resulting in inhibition of the AKT/mTORC1 cascade downstream of PI3K (Fig. 5).

Since mTORC1 controls processes in which amino acids play a fundamental role, such as ribosome biogenesis and mRNA translation, it is favorable for the cells to be able to respond to variations of the levels of essential amino acids that mammalian cells are unable to synthesize themselves. Leucine is a strong activator of mTORC1. The mechanisms by which leucine and the other amino acids activate the pathway are still not clear but the downstream effects of mTORC1 activation are well studied and include phosphorylation of S6K1 and 4E-BP1 (115). Proteins such as class III phosphoinositide 3-kinase (human vacuolar protein sorting 34), Rag-GTPases and MAP kinase kinase kinase kinase 3 (MAP4K3) have been proposed to act as intracellular sensors of these molecules (116).

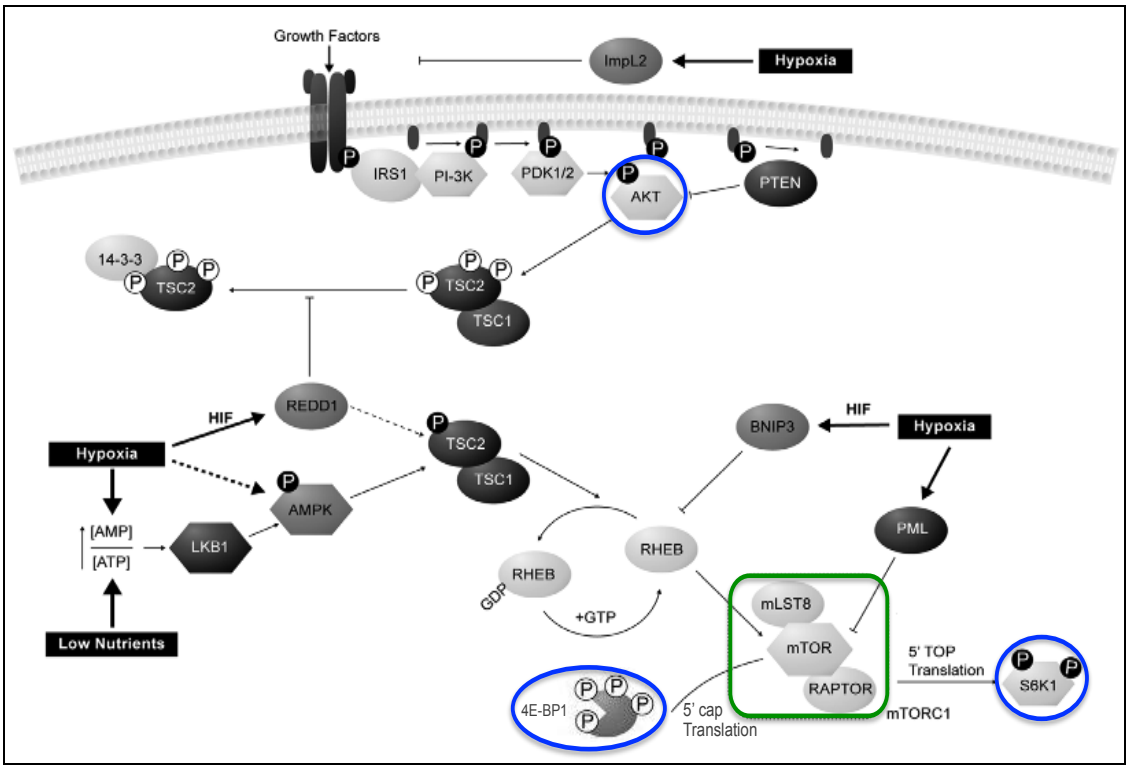

Fig. 5 Mammalian target of rapamycin complex 1 (mTORC1) signaling pathway. Growth factors, nutrients, oxygen availability promote mTORC1 signaling via phosphorylation cascade. Activation of the PI3K/AKT signaling converges on TSC1/2 complex inhibition, leading to Rheb activation. Subsiquently phosphorylated mTORC1 promotes cap and TOP-dependent translation, via 4E-BP1 and S6K1 phosphorylation respectively. Stimulating (*white P in black circle*) and inactivating (*black P in white circle*) phosphorylations are indicated. mTORC1 is illustrated in a green square while blue circles indicate proteins analyzed in this thesis work. Figure modified from (47).

4.3.2.2 Regulation of mTORC1 by cellular energy and oxygen

Translation and ribosome biogenesis are the most energy-costly biosynthetic processes in the cells (117), therefore these mechanisms must be finely regulated during periods of reduced energy charge (i.e. rise in [AMP]/[ATP] ratio) or O_2 availability.

Energy depletion inhibits mTORC1 via AMPK and involves the activation of TSC1/2 complex (118). High levels of AMP (low cellular energy charge) trigger, through direct binding, the activating phosphorylation of AMPK by LKB1 (119). Activated of AMPK then phosphorylates and stimulates TSC1/TSC2 inhibitory function towards Rheb and mTORC1 (118), in contrast to the inactivating phosphorylation triggered by AKT in response to growth factors (Fig. 5). Inhibition of mTORC1 via TSC1/TSC2 complex during low energy charge reduces the rate of protein synthesis to rematch declining ATP supplies with decreasing ATP demand.

Multiple pathways converge on mTORC1 during hypoxia. However since O_2 availability ultimately regulates ATP in the cells, it is not surprising that effect of O_2 deprivation, to some extent, are similar to those caused by energy depletion. Under conditions of mild hypoxia elevated [AMP]/[ATP] ratios inhibit mTORC1 signaling via AMPK-driven TSC1/2 activation (120, 121), as previously described. Yet, O_2 deprivation itself is also able to negatively regulate mTORC1 activity by promoting the TSC1/2 interference of the kinase through the HIF-1-driven induction of the REDD1 (regulated in development and DNA damage responses 1) gene product (122). Indeed, REDD1 (aka DDIT4, RTP801) releases the subunit TSC2 from its growth factor-induced association with inhibitory 14-

3-3 proteins (123) therefore activating TSC1/2 complex and blocking mTORC1 signaling. Additionally, it was recently shown that during hypoxic conditions Bcl-2/adenovirusE1B19-kDa interacting protein 3 (BNIP3) disrupts the interaction between the kinase subunit mTOR and its positive regulator Rheb, thus inhibiting mTORC1 (Fig. 5).

4.3.3 Downstream of mTORC1

Notably, active mTORC1 triggers mRNA translation by directly stimulating phosphorylation of S6K1 and inactivating phosphorylation of 4E-BP1 to ultimately stimulate 5-TOP- and 5'-cap-dependent mRNA translation, respectively (Fig. 6). The entire mRNA translation process consists of initiation, elongation and termination stages, of which 4E-BP1 and S6K1 regulate the first stage (initiation), which is considered to be the rate-limiting step of the whole mechanism (124). Each of these effectors and corresponding mechanisms will be discussed in more detail below.

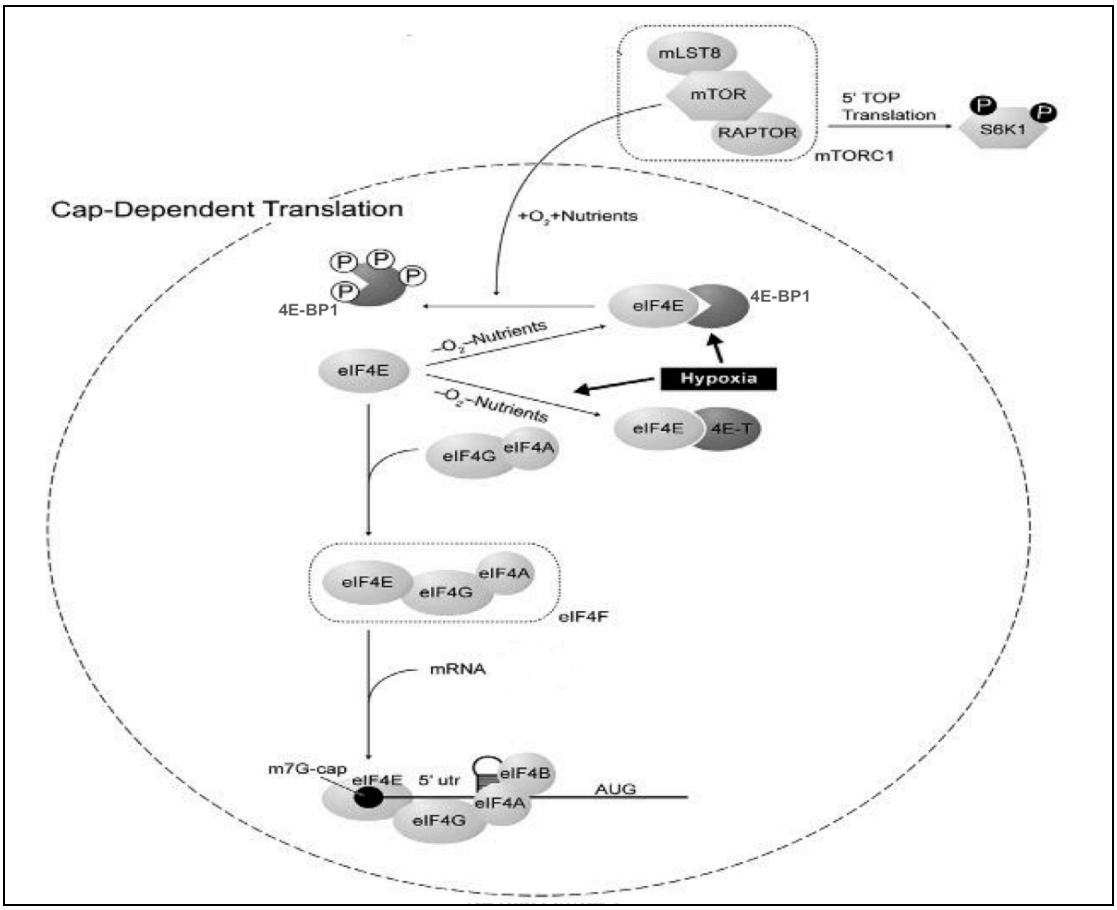

Fig. 6 Regulation of translation by mTORC1. Nutrients and O_2 availability activate mammalian target of rapamycin (mTOR) complex 1 (mTORC1), which phosphorylates eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 70kDa (S6K), therefore activating cap and TOP-dependent translation. Full 4E-BP1 hyper-phosphorylation results in the release of eIF4E, which recruits eIF4G and eIF4A to constitute eIF4F complex. The eIF4F interacts with the cap structure at the 5' terminus of the mRNA and facilitates recruitment of other factors to initiate the cap-dependent translation. During nutrient starvation and/or hypoxia, hypo-phosphorylated 4E-BP1 interacts with eIF4E and impedes the formation of eIF4F, while eIF4E-T triggers the translocation of eIF4E, triggering cap-dependent translation inhibition. Figure modified from (47).

4.3.3.1 5'-cap-dependent protein synthesis

Most eukaryotic mRNAs possess a m^7GpppN (where m: methyl group; G: guanine base; ppp: triphosphate group; N: any nucleotide) "cap" structure at their 5' terminus. This modification is essential in determining the life span of the mRNA as it affects splicing, transport, stabilization and translation of the transcript (125). The cap structure facilitates recruitment of the nascent mRNA to the eukaryotic initiation translation factor 4F (eIF4F) complex and 40S ribosomal subunits (126, 127). The eIF4F complex consist of eIF4E, eIF4G and eIF4A (126). eIF4E directly binds the 5' cap structure of the mRNA and coordinate recruitment of eIF4G and eIF4A onto the nascent transcript (128). The eIF4E/cap interaction is the most controlled target of the process (126) and is considered to be the first rate limiting step of translation. eIF4A, a member of the DEAD box helicase family, unwinds secondary structures of the mRNA to facilitate the scanning of the 40S ribosomal subunit for the initiation codon. The eIF4Gs (i.e. eIF4G1, eIF4G2) are scaffolding proteins that interact with eIF4E. Through this direct association with eIF4E, the eIF4G factors co-localize the proteins implicated in the translation initiation mechanism with 43S pre-initiation complex, which consists of the small 40S ribosomal subunit, the initiation factor eIF3, and the ternary complex eIF2-GTP-$tRNA^{Met}$ (129).

The second most important check point for the initiation of the mRNA translation is the assembly of the eIF2-GTP-$tRNA^{Met}$ complex, catalyzed by eIF2B. Phosphorylation of the eIF2α subunit at serine-51 (S51A) residue by the PKR-like ER-associated kinase (PERK) (130) inhibits eIF2B and blocks initiation of protein synthesis upon accumulation of unfolded or misfolded proteins within the endoplasmic reticulum (i.e. unfolded protein response) (131). Regulation of

mRNA translation either via eIF4F assembly or eIF2α phosphorylation seems to be coordinated. Indeed, a recent study on HeLa cells has shown that hypoxic inhibition of mRNA translation is a biphasic process characterized by very fast, yet transient phosphorylation of eIF2α, which is followed at later times by a more persistent suppression of the eIF4F assembly (83). This biphasic regulation of the translation initiation is of particular interest for our study as we wanted to investigate the translational response of brain cells exposed to prolonged O_2 deprivation, i.e. at times when the mechanism presumably is interfered with via eIF4F dissociation.

Availability of eIF4E, and of the translation-promoting eIF4E/eIF4G partnering, depends on finely regulated mechanisms of interaction of the eIF4E subunit with inhibitory binding partners. Over the last years proteins such as Maskin, Cup, Neuroguidin and CYFIPI have been shown to be inhibitory partners of eIF4E (129), however the three members of the 4E-BP family seem to play the major role in antagonizing cap-dependent translation via their scavenging of eIF4E.

4.3.3.1.1 4E-BP mediated inhibition of 5' cap-dependent translation

The eIF4E binding proteins (4E-BPs) are a group of proteins encoded by three paralogous genes: 4E-BP1, 4E-BP2, 4E-BP3 (132). The 4E-BPs show similar inhibitory activity on mRNA translation (133), however 4E-BP1 has been characterized the most. Interaction of 4E-BP1 with eIF4E is a reversible mechanism dependent on the phosphorylation state of the binding protein (Fig. 6). Hypo-phosphorylated 4E-BP1 competes with eIF4G for an overlapping binding site in eIF4E. Thus, the interaction of 4E-BP1 with eIF4E impede the

formation of eIF4F complex and the recruitment of the 40S ribosome for the translation initiation (134). A hierarchical cascade of phosphorylation reactions at multiple sites regulates 4E-BP1 capacity to interact with eIF4E. Fully phosphorylated (hyper-phosphorylated) 4E-BP1 can no longer retain the binding of the cap-binding factor, which results in the release of eIF4E and the initiation of the cap-dependent translation. Different pathways such as PI3K, AKT and mTORC1 regulate 4E-BP1 phosphorylation (135). Indeed, hyper-phosphorylation of 4E-BP1 has been shown to be triggered by stimulated PI3K and AKT/PKB (protein kinase B) signaling (136). mTORC1 itself has also been shown to directly phosphorylate 4E-BP1 (135).

Human 4E-BP1 contains seven phosphorylation sites: Thr37, Thr46, Ser65, Thr70, Ser83, Ser101 and Ser112. However, not all the sites are equally responsible for 4E-BP1 regulation. Recent data by Gingras et al. demonstrated that phosphorylation of Thr37 and Thr46 via mTORC1 are priming events necessary to trigger the final phosphorylation reactions of Thr70 and Ser65 (137). Moreover, the authors showed that phosphorylation of Ser65, alone or in combination with Thr70, does not interfere with 4E-BP1/eIF4E binding, therefore suggesting that phosphorylation at multiple sites is required for the disruption of such binding.

4.3.3.1.2 4E-BP mediated physiological and pathophysiological functions

Since 4E-BPs directly control the initiation of cap-dependent translation, it is not surprising that they play a fundamental role in many physiological and pathophysiological mechanisms. 4E-BP2 is mainly expressed in the brain (138)

where is required for synaptic plasticity, spatial learning and memory (139). In keeping with this, 4E-BP2 knockout mice exhibit an autistic-like phenotype (140), thereby indicating a fundamental role of this binding protein in establishing correct cell-cell connections in the brain. Regarding 4E-BP1, it is clear to date that this isoform is critical in numerous physiological mechanisms, including regulation of adipogenesis and metabolism (138), as well as control and synchrony of the circadian clock (141). Whereas insulin stimulates 4E-BP1 phosphorylation in proximal tubular epithelial cells (142), low sulfur amino acid intake is known to increase 4E-BP1 expression in liver of rats (143). However, 4E-BP1 is also described as key effector in many cancer entities (144–146). Activation of cap-dependent protein synthesis by chronic hyper-phosphorylation of 4E-BP1 is associated with increased cancer progression and decreased survival in astrocytoma (146). Similarly, it was shown that constitutive hyper-phosphorylation of 4E-BP1 results in increased active eIF4E, therefore aberrant translational control, in human breast carcinoma cell lines (147). Even decreased levels of the binding protein have been associated with cellular abnormalities due to hyper-activation of eIF4E. Indeed, silenced expression of 4E-BP1 was found to i) increase resistance to Enzastaurin (acyclic bisindolylmaleimide)-induced apoptosis in human glioma and colon cancer cell lines (148), ii) to sensitize U87 glioblastoma xenograft tumors to experimental irradiation by decreasing hypoxia tolerance (149). Moreover, 4E-BP1 levels have been found to inversely correlate with the progression of gastrointestinal cancer (150).

It thus emerges that 4E-BP proteins play a fundamental role in many physiological mechanisms, and that alteration in expression levels or

phosphorylation state may affect activities of normal cells as they do for cells with malignant background.

4.3.3.2 S6K1 and TOP-dependent translation

S6Ks (S6K1 and S6K2) are members of the AGC family of serine/threonine kinases and control translation of mRNA containing a 5' terminal oligopyrimidine (TOP) region. S6K1 is expressed as two isoforms (p85 and p70) that derive from the same transcript through alternative translation initiation and are primarily localized in the cytoplasm (151). In contrast, the two S6K2 isoforms (p54 and p56) are mainly nuclear (152). Although the specific roles of S6K1 and S6K2 are still poorly understood, a recent study indicates S6K1 to be ubiquitously present, while S6K2 levels vary among different tissues (153). With regard to the better known S6K1 isoform, it is clear that, Thr389 and Thr229 function as essential sites for the activation of the kinase, despite the fact that several additional sites may also be phosphorylated (154). mTORC1 mediates phosphorylation of Thr389, followed by the PDK1-driven phosphorylation at Thr229 (154). Active S6K1 eventually phosphorylates the S6 protein of the 40S small ribosomal subunit, which, in turn, initiates the translation of TOP-dependent mRNAs. This mRNA category mainly encodes components of the translational machinery, e.g. ribosomal protein S6 and eukaryotic initiation factor 4B (eIF4B). Other phosphorylation targets of active S6K1 are IRS1, glycogen synthase kinase 3 (GSK3), eIF4B, translation elongation factor 2 (eEF2), and Bcl-2-associated death promoter (Bad) (155). So far, regulation of IRS1 via S6K1 broadly attracts research due to its influences on the insulin cascade.

Indeed, phosphorylation of IRS1 via S6K1 interferes with the interaction of the substrate with the insulin receptor, thus inhibiting insulin signaling (156). Although this negative feedback loop serves to down-regulate insulin stimulation of the PI3K cascade, its chronic activation may contribute to diabetes (157).

4.3.4 Upstream and downstream of mTORC2

In addition to the activation of mTORC1, growth factors such as insulin and IGF1 also stimulate mTORC2 activity. Although the mechanisms still remain unclear, it was recently suggested by Huang et al. that the TSC1/TSC2 complex, inhibitor of mTORC1, mediates the activation of mTORC2 via direct binding (158). However, the GTPase Rheb, downstream effector of TSC1/TSC2 complex and direct activator of mTORC1 (see section 4.3.2.1), does not appear to play a role upstream of mTORC2 (158). Although little is known about mTORC2's upstream regulation, upon activation the complex phosphorylates members of the AGC kinase family including SGK1 (serum and glucocorticoid induces protein kinas 1) (159), PKC (protein kinase C) (160) and AKT (161), therefore triggering different cellular mechanisms. The mTORC2 mediated phosphorylation of SGK1 at Ser422 contributes to the maintenance of sodium homeostasis via stabilization of the ENaC (epithelial sodium channel) (162), while PKC phosphorylation is involved in the regulation of the actin cytoskeleton (163). AKT phosphorylation (112, 161) is considered to be one of the most critical functions of mTORC2 complex. Indeed, phosphorylation of AKT at the Thr450 and Ser473, mTORC2 contributes to stability and activation of this important cell signaling kinase. It is widely accepted that mTORC2 mediated phosphorylation of AKT at Ser473 fully

activates the kinase in the PI3K/ AKT cascade (see section 4.3.2.1)(112). However, it was recently shown by Facchinetti et al that the mTORC2 complex also controls the phosphorylation of AKT at the Thr450, thereby regulating folding and stability of this kinase (160). The importance of the mTORC2 complex is revealed by the lethal phenotype during embryonic development of mice lacking *rictor* or *SNI1* (see section 4.3.1) (104, 164). A recent *in vivo* study provides evidence that the mTORC2 complex affects hepatic gene expression, transcriptional regulation and metabolism (165), therefore playing diverse biological roles. Indeed it was shown by Urbanska et al that together with mTORC1 the mTORC2 complex is needed for the correct dendritic morphology of *in vitro* neurons (166). Interestingly, mTORC2 seems to play a role also in pathophysiological processes as mTORC2 activity was recently discovered to be crucial in ischemic preconditioning-induced cardioprotection (167). Moreover, due to the role of this kinase in regulating cytoskeleton rearrangement, it is not surprising that mTORC2 plays a role in different aspects of cancer progression. Gene silencing of Rictor, a fundamental component of the mTORC2 complex (see section 4.3.1), decreased cell migration and invasion of *in vitro* and *ex vivo* model of human bladder cancer cells (168), while tumor cells lacking Rictor failed to form tumor xenograft (169). Specific pharmacological inhibition of the complex also prevented breast tumor growth in nude mice (170). It is therefore clear that although mTORC1 is the most studied mTOR dependent complex, also mTORC2 plays a fundamental role in many different cellular mechanisms.

Regulation of protein synthesis is fundamental for the adaptation to O_2 deprivation. Adjusting mTORC1 signaling to the altered environment clearly

plays a major role for this adaptation. With this study we aimed to investigate how mTORC1 pathway is modulated in primary O_2 sensitive (i.e. neurons) and tolerant (i.e. astrocytes) brain cells and which role it occupies in controlling overall protein synthesis and energy steady states. Identification of mechanisms regulating the very distinct O_2 sensitivities between nerve and glia cells could be beneficial for the discovery of new biological targets for pharmacological treatment of hypoxia-driven CNS injuries.

5 Aim

Decreased protein synthesis is considered a first common cellular mechanism that cells undergo to reduce energy consumption during hypoxic insult (82), although transcriptional regulation, particularly via HIFs, also plays a major role. The mTORC1 pathway is fundamental in cell stress responses since it controls protein synthesis via ribosomal biogenesis and translation of most mRNAs (123, 171). Furthermore published data suggests that mTORC1 inhibition impacts HIF-1α signaling and thus transcriptional and translational control may collaborate in the complex mechanisms stimulated during hypoxia. Although mTORC1 has been extensively studied in cancer cells, only recent studies highlighted its role in regulating physiological brain development (172), and synaptic plasticity (173).

The aim of this thesis work was to understand the role of mTORC1 signaling, and particularly 4E-BP1, in astrocytic and neuronal responses to oxygen and/or glucose deprivation. *Specifically, we hypothesized that the mTORC1 cascade is responsible for differential regulation of overall protein synthesis and energy levels in neurons and astrocytes challenged with hypoxia or ischemia, respectively*. Additionally, we investigated whether mTORC1 influences transcriptional and translational mechanisms during hypoxic response in primary brain cells by modulating HIF-1α stabilization. To this aim we subjected primary neurons and astrocytes to different severities of oxygen and/or glucose deprivation. Subsequently we investigated regulation of the mTORC1 effectors S6K1 and 4E-BP1, HIF-1α as well as translational responses including protein synthesis rate and cell survival.

6 Materials and methods

Methods not listed below are described in section 10 (Manuscript).

Primary culture of neurons

Primary neuronal cultures were obtained from the cerebral cortex of C57B1/6J mice (gestational stage E14) as described previously (174). Dissected cortices were dissociated in Hank's buffered salt solution (HBBS) containing trypsin and DNase I for 5 min at 37°C. Neurons were seeded in poly-L-lysine coated petri dishes (3 x 10^6 cells per 100mm dish), in neurobasal medium complemented with B27 supplement (1X), AlbuMAXI (0.25g/ml), streptavidin-penicillin (1%), sodium-pyruvate 100U/ml and L-glutamine (0.5 mM). Neurons were maintained in culture for 6 days (days *in vitro* =DIV) at normal atmosphere (21% O_2) in a humidified incubator at 37°C. For analysis of culture purity, immunofluorescence staining was performed on 6 DIV neurons using the astrocyte marker GFAP and the neuronal marker NeuN (neuronal nuclear antigen). 6 DIV neurons were estimated to be 98% pure.

PI staining

Cell survival was determined by propidium iodide (PI) staining using an automated cell counter (ADAM, Digital Bio) according to manifacturer's instructions. Following incubation cells were trypsinized, stained with PI and counted. Percentage of cell death was determined as ratio of the number of positively stained cells compared to total number of cells.

LDH assay

Cytotoxicity was determined by lactose dehydrogenase (LDH) cytotoxicity assay kit (Roche, Penzberg, Germany) according to manifacturer's instructions. Following incubation, cell culture media was transferred to a 96 wells/ plate, reaction mixture was added (1:1 v/v) and incubated for 30 min at room temperature in the dark. Absorbance was measured at 490 nm with reference filter at 650 nm.

ATP measurements

ATP levels were measure by an ATP biomiluminescent assay kit (Sigma, Buchs, Swizterland) and used according to manifacturer's instructions. Following incubation cell lysates were generated using cell lysis buffer supplemented with protease inhibitor cocktail and sodium orthovanadate (1 mM), incubated 10 min on ice and then cleared by centrifugation for 10 min at 16,000 x g at 4°C. The supernatant was mixed with 5% TCA (1:1 v/v) to block residual ATPase activity and luminescence was measured using Berthold luminometer (Detection system GmbH, Pforzheim, Germany). ATP amount were determined from a standard curve.

MTT

Mitochondrial activity was measured by MTT (Sigma-Aldrich, St. Louis, MO) assay. After exposure a solution of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to the medium (final concentration 0.5 mg/ml) and cells were incubated for 1 hour at 37°C. The MTT-containing medium was then removed and purple formazan crystals dissolved

by adding DMSO (Dimethyl Sulfoxide). Optical density was measured at 560 nm with reference filter at 670 nm.

7 Own research

7.1 ASTROCYTIC RESPONSE TO ACUTE (16 HOURS) OXYGEN DEPRIVATION

The following research represents the basis of a submitted manuscript (see section 10, accompanying manuscript).

7.1.1 4E-BP1 and S6K1 have different sensitivity to mTORC1 inhibition

Immunoblot analysis shows that 4E-BP1 and S6K1, the two downstream effectors of the mTORC1 kinase, are differently sensitive to inhibition of the pathway. Despite the fact that ischemia completely dephosphorylated both S6K1 and 4E-BP1, treatment with rapamycin and 16h anoxia were effective in abrogating S6K1 phosphorylation while these applications only partially hypo-phosphorylated 4E-BP1. Different studies have shown that the kinase AKT can be activated by rapamycin-driven S6K1 dephosphorylation (175) and that this signaling cascade can mediate 4E-BP1 regulation (136). Under our conditions rapamycin treatment increased AKT phosphorylation, thus activated AKT may in turn sustain 4E-BP1 phosphorylation at times when mTORC1 is inhibited.

7.1.2 Hypo-phosphorylated 4EBP1 scavenges eIF4E but does not affect overall protein synthesis

Since partial hypo-phosphorylation of 4E-BP1 by rapamycin treatment barely affected the assembly of the initiation complex, incomplete dephosphorylation of the binding protein appears inadequate to inhibit cap-dependent translation.

It was shown that 4E-BP1 in immortalized breast epithelial cells at least partially contributes to the regulation of overall protein synthesis during oxygen deprivation (176). However, in primary astrocytes, where general mRNA translation decreased with oxygen deprivation, the hypo-phosphorylation of 4E-BP1 during 16h ischemic challenges or rapamycin application did not further intensify inhibition of global protein synthesis compared to the anoxic decrease, where hyper-phosphorylated 4E-BP1 prevails.

7.2 ASTROCYTIC RESPONSE TO PROLONGED (36 HOURS) OXYGEN DEPRIVATION

7.2.1 Astrocytic survival is not affected by prolonged oxygen and/or glucose deprivation

To increase the challenge of these stress-resistant cells, we prolonged the oxygen deprivation and drug treatment to 36 hours and investigated cell death by propidium iodide (PI) staining (Fig. 1). As expected, astrocytes were not affected by mild oxygen deprivation (Hx, 1% O_2) and 10 nM rapamycin treatment, while 36 hours near-anoxic exposure showed a trend to increase cell death to almost 20%. Interestingly, however, simultaneous glucose withdrawal and severe oxygen deprivation did not further affect astrocytic survival. Altogether these data indicate that astrocytes are highly resistant to stress conditions, although prolonged and severe oxygen deprivation seem to marginally reduce cell survival.

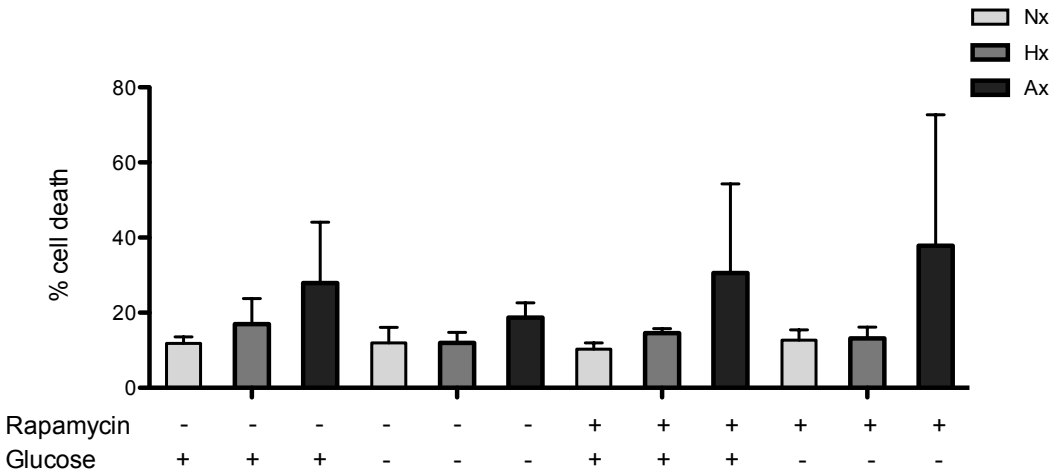

Fig. 1 Astrocytic survival is not affected by prolonged oxygen and/or glucose deprivation. After 36h exposure, astrocytes show a trend to increase cell death only under near-anoxic condition. Glucose deprivation and rapamycin treatment do not affect astrocytic survival. Values are means ± SD (n=3; 36h Ax-G n=2).

7.2.2 Differential effect of Rapamycin-driven mTORC1 inhibition on 4E-BP1 and S6K1 phosphorylation

To investigate the regulation of mTORC1 pathway we analyzed the changes in 4E-BP1, eIF2α, S6K1 and AKT phosphorylation by immunoblot. Oxygen or glucose deprivation alone did not alter astrocytic 4E-BP1 phosphorylation relative to the Nx±G control situation (Fig. 2). However, hypoxia together with glucose withdrawal moderately reduced phosphorylated 4E-BP1 (stronger β band) and ischemia completely inhibited it (α band; Fig. 2), parallel to increased eIF2α phosphorylation, as observed in preliminary studies (data not shown). As previously observed (see section 7.1.1), rapamycin treatment only partially hypo-phosphorylated 4E-BP1 (γ, β, α bands). S6K1 phosphorylation, on the other hand, was completely inhibited during ischemia and drug treatment, while oxygen or glucose deprivation alone was less effective (Fig. 2). AKT was activated during 36 hours rapamycin treatment (elevated p-AKT levels), while ischemia inhibited it. These results mimic our previous data on 16 hours exposure, indicating that prolonged stress has no additional effect on phosphorylation of 4E-BP1 and S6K1 and that AKT may indeed contribute to the phosphorylation of the binding protein also during prolonged rapamycin treatment.

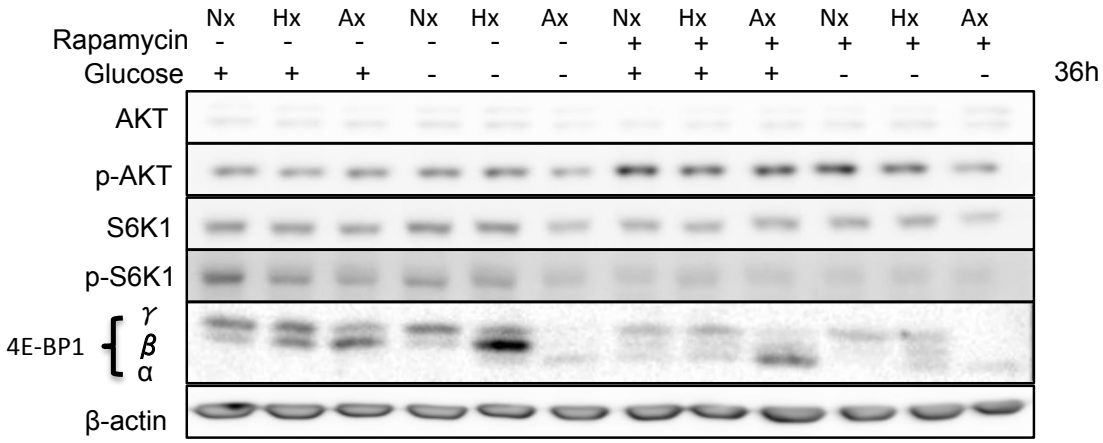

Fig. 2 Rapamycin treatment partially inhibits 4E-BP1 phosphorylation and completely dephosphorylates S6K1. Representative Western blot analysis of n=3 of independent experiments shows phosphorylated (p-) and total levels of S6K1 and AKT and phosphorylation states of 4E-BP1 (γ, β and α bands). β-actin was used as loading control.

7.2.3 Rapamycin-dependent 4E-BP1 hypo-phosphorylation affects protein synthesis rate only in normoxic astrocytes exposed to glucose deprivation

To determine if varying degrees of 4E-BP1 phosphorylation impact global mRNA translation in astrocytes, we investigated overall protein synthesis rates after 16h (see section 10, accompanying manuscript) or 36h incubations as described in this section (Fig. 3). Compared to normoxic controls (Nx), overall protein synthesis rates dropped progressively with increasing O_2 deprivation. 36 hours hypoxia (Hx) reduced the rate of protein synthesis to 65% of the control ($p<0.05$), while anoxia further reduced it to 35% ($p<0.001$). Glucose withdrawal had no additional effects during hypoxic exposure, yet, for unknown reasons, marginally stimulated protein synthesis in aerobe glia cells (Fig. 3). *In vitro* ischemia (Ax-G), however, caused the expected massive slowdown of global mRNA translation (5% of controls; $p<0.0001$). Treating astrocytes with 10 nM rapamycin in combination with glucose deprivation reduced translational rate to 67% in normoxia glia ($p<0.05$), but did not further attenuate protein synthesis rates in the other conditions (comparing same condition, with versus without rapamycin). These data suggest that dephosphorylated 4E-BP1 (36h Ax-G in Fig. 2) may contribute to the massive inhibition of protein synthesis rate during prolonged ischemia. Yet, the incomplete 4E-BP1 hypo-phosphorylation following 36h rapamycin application (Fig. 2) reduces total mRNA translation only in normoxic astrocytes challenged with glucose withdrawal, while it does not affect protein synthesis in cells exposed to oxygen deprivation.

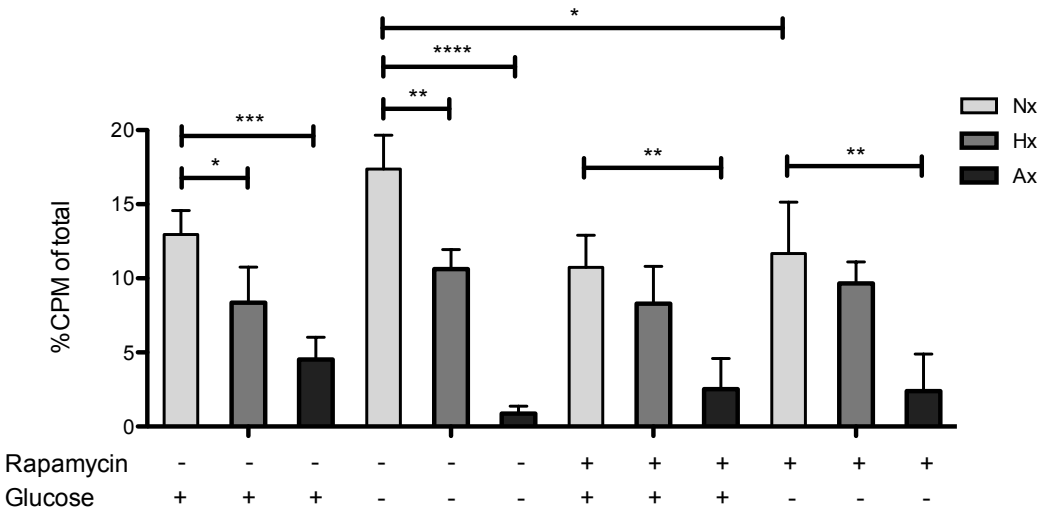

Fig. 3 Rapamycin-dependent 4E-BP1 hypo-phosphorylation affects protein synthesis rate only in normoxic astrocytes exposed to glucose deprivation. In 36h incubations overall protein synthesis rates decrease as a function of O_2 deprivation but are not additionally inhibited by glucose deprivation. 10 nM rapamycin treatment reduces mRNA translation only when astrocytes are exposed to normoxia in absence of glucose. Values are means ± SD (n=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

7.2.4 Ischemia severely reduces ATP but does not affect MTT levels

We have shown that in primary astrocytes global translation decreases with oxygen deprivation but rapamycin treatment does not further reduce it (see section 10, accompanying manuscript). Since ATP steady state is closely linked with translational regulation, we investigated cellular energy regulation after both 16 and 36 hours exposure (Fig. 4). 16 hours oxygen and glucose deprivation alone did not affect ATP levels, while ischemia resulted in approximately 50% decrease ($p<0.05$) that was not reversed by rapamycin treatment (Fig. 4A). 36 hour of glucose withdrawal during normoxia elicited a surprising 50% ($p<0.05$) increase in energy content, while ATP was completely depleted during anoxic conditions ($p<0.0001$). The effects of oxygen ± glucose deprivation during prolonged exposure were not reversed by drug treatment (Fig. 4B). These data clearly highlight the susceptibility of ATP steady state levels only to severe stress conditions and that prolonged mTORC1 inhibition and 4E-BP1 dephosphorylation does little to prevent ATP depletion. Activity of mitochondrial dehydrogenases is not affected by oxygen deprivation either after 16 hours (Fig. 5A) or 36 hours (Fig. 5B) exposure. Although glucose withdrawal caused a general trend of MTT concentrations to fall, it is interesting to note that only the combination with rapamycin treatment significantly reduced its levels to 50-60% ($p<0.05$) (Fig. 5A&B), suggesting a possible role for mTORC1 in stimulating substrate turnover in mitochondria.

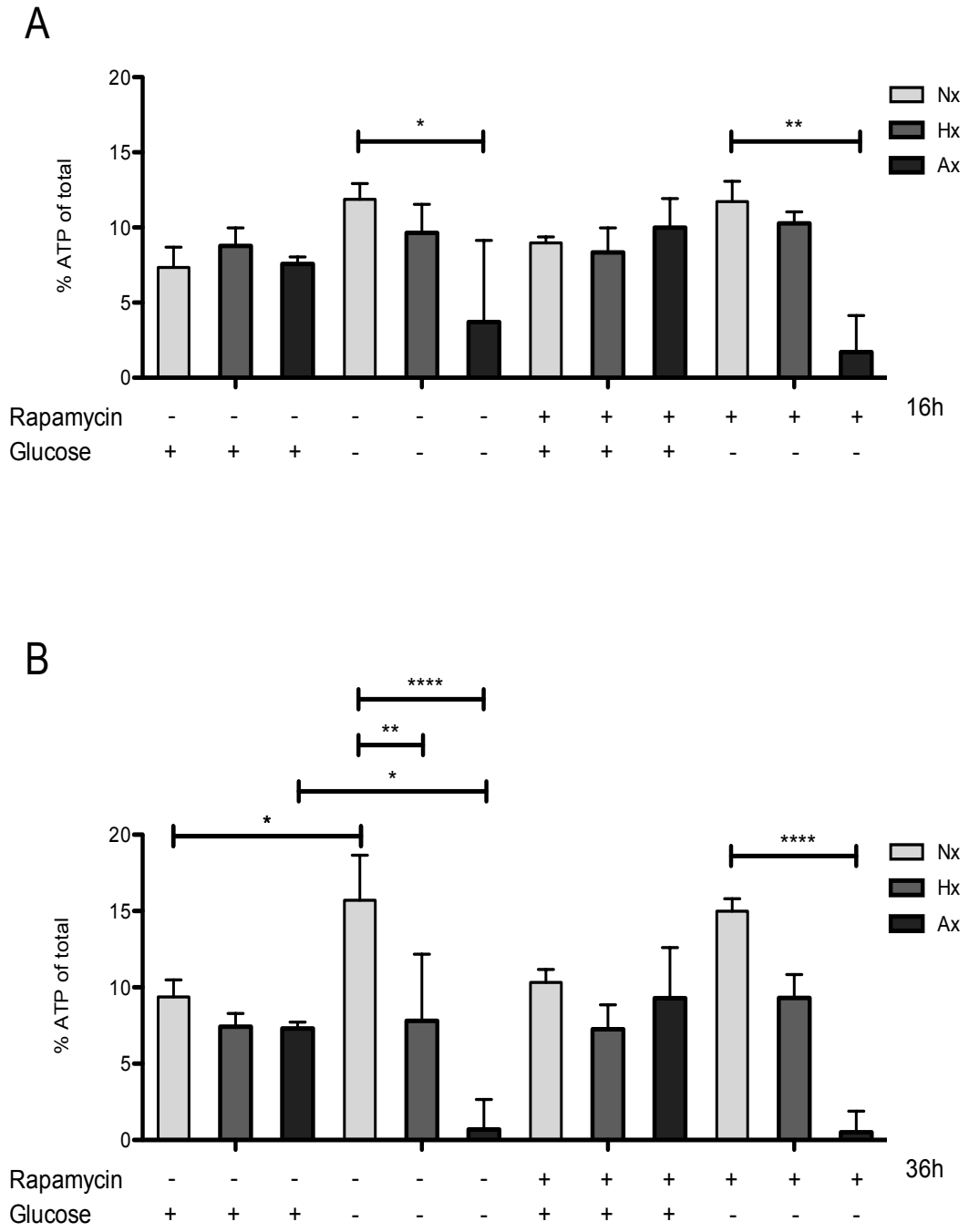

Fig. 4 Ischemia severely reduces ATP levels. Oxygen and glucose deprivation lead to a dramatic drop in ATP levels after both 16 A) and 36 B) hours incubation and 10 nM rapamycin treatment did not rescue it. Values are means ± SD (n=3). *p<0.05, **p<0.01, ****p<0.0001

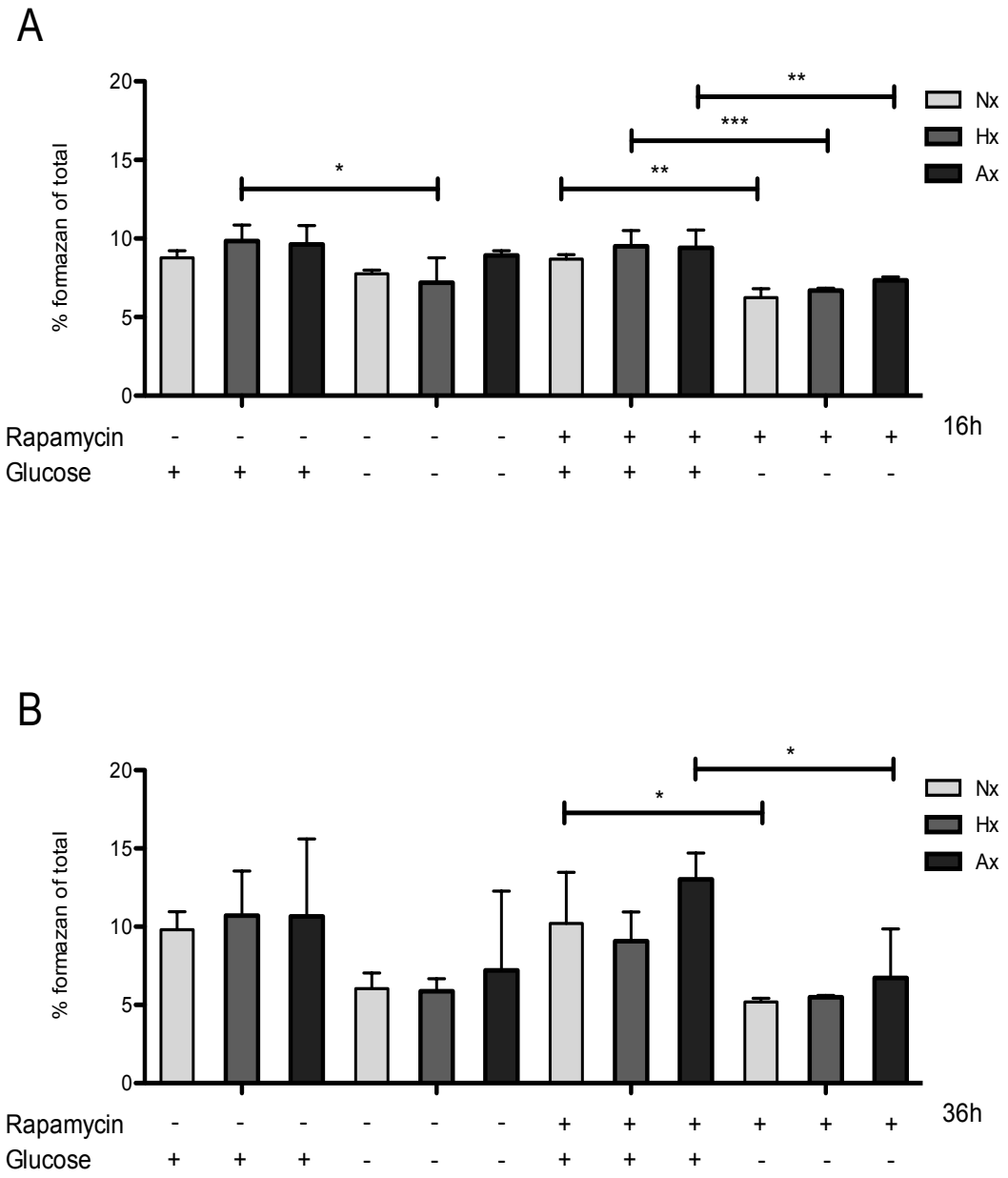

Fig. 5 Ischemia does not affect MTT levels. Oxygen and glucose deprivation affect MTT levels only when cells are treated with 10 nM rapamycin after either shorter A) and prolonged B) time exposures. Values are means ± SD (n=3). *p<0.05, **p<0.01, ***p<0.001

7.2.5 Rapamycin reduces HIF-1α stabilization during oxygen deprivation

In agreement with recent evidence on the crosstalk of HIF-1α and mTORC1 signaling (177), our previous data on neuronal cells indicated a correlation between decreased HIF-1α stabilization and rapamycin exposure. In astrocytes 16 hours oxygen deprivation also stabilized HIF-1α, while glucose withdrawal did not add to the accumulation of the HIF-1α subunit (Fig. 6A). Interestingly, rapamycin treatment caused an attenuation of HIF-1α levels (Fig. 6A). Similar results were obtained even after prolonged (36h) exposure although total levels of HIF-1α were reduced compared to 16 hours exposure, which probably reflects the transient nature of HIF-1 induction during periods of O_2 scarcity (Fig. 6A&B). Overall, we provide strong evidence that crosstalk between the mTORC1 cascade and the hypoxic response regulator in O_2-deprived primary astrocytes occurs.

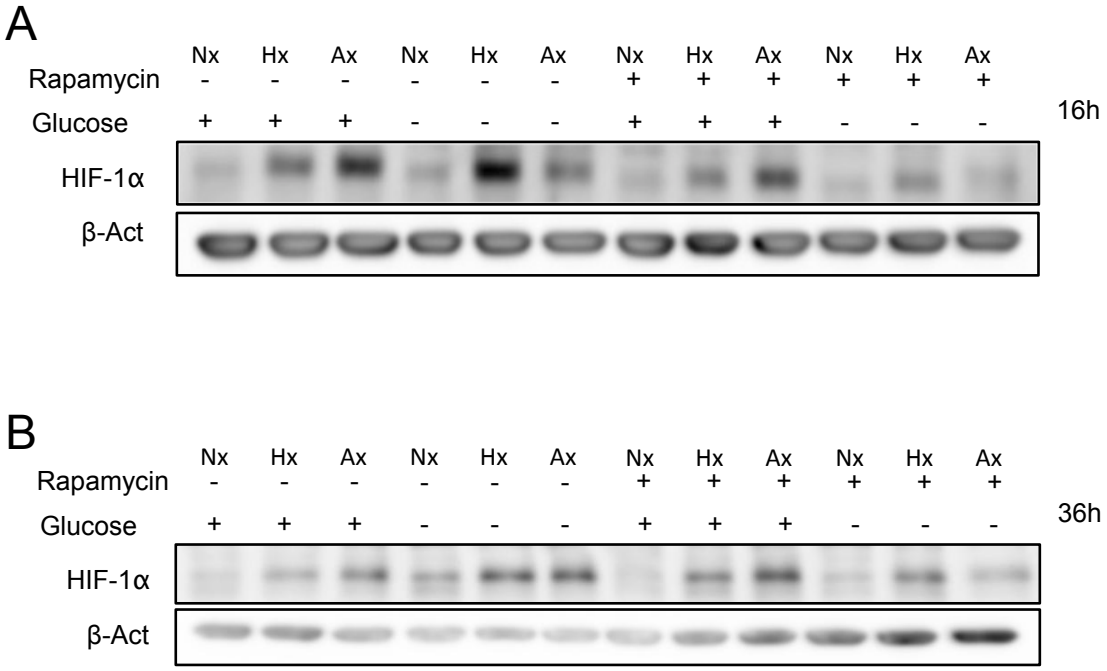

Fig. 6 Rapamycin reduces HIF-1α stabilization during oxygen deprivation. Representative Western blot analysis of n=3 independent experiments shows HIF-1α levels after 16 (Fig 6A) and 36 (Fig 6B) hours exposure to oxygen deprivation and 10 nM rapamycin. β-actin was used as loading control.

7.2.6 4E-BP1 is the major player in the astrocytic response to severe stress

Interestingly, in primary astrocytes grown *in vitro* 4E-BP1 protein level expression clearly dominated over abundance of 4E-BP2 and showed sensitivity towards changes in the supply of oxygen and glucose (Fig. 7). As noted before, 4E-BP1 was completely dephosphorylated during ischemia (α band), while 4E-BP2 was refractory to it. Moreover, rapamycin treatment reduced phosphorylation of 4E-BP1 (γ, β, α bands) but not of 4E-BP2. In section 7.4.2 we show that 4E-BP2 is the isoform preferentially expressed in neuronal cells, although we could not detect any O_2-responsive regulation of this binding protein under our conditions. These data indicate that 4E-BP1 is the predominant and O_2/glucose-sensitive isoform in primary astrocytes.

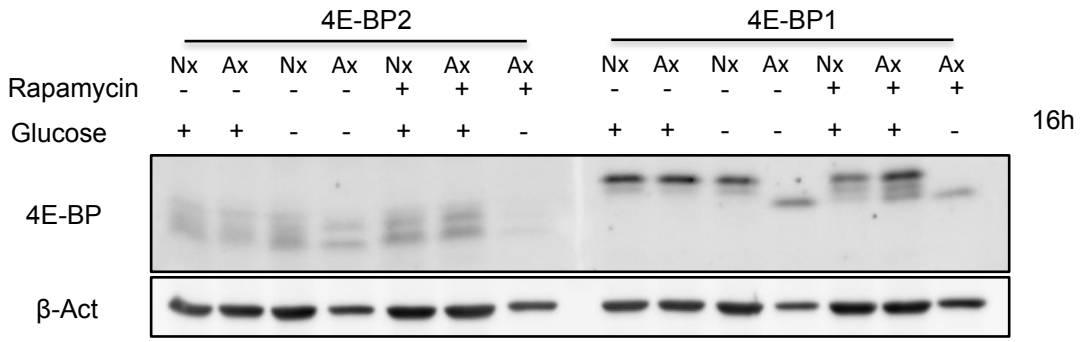

Fig. 7 4E-BP1 is the major player in the astrocytic response to severe stress. Representative Western blot analyses of n=2 independent experiments reveals that in astrocytes 4E-BP1 is more expressed than 4E-BP2 and is regulated in O_2/glucose-sensitive manner. β-actin was used as loading control.

7.3 DISCUSSION ON ASTROCYTIC RESULTS

Astrocytic cells are capable of withstanding hypoxic and ischemic injury for long periods of time (178). Hypoxic and ischemic insults stimulate proliferation of resident astrocytes *in vivo* (26, 40, 179), a process called astrogliosis. Although it was shown that VEGF signaling plays an important role in astrocytic proliferation and resistance to oxygen and glucose deprivation *in vitro* (23), additional mechanisms endowing astrocytes with this remarkable degree of stress tolerance and its overall meaning for the highly susceptible neurons still need to be fully elucidated. mTORC1 has been shown to orchestrate energy requiring synthetic and proliferative responses with ambient oxygen deprivation in different human cancer cells (93) and to play a role in astrocytic activation during CNS injury (180). Inhibition of mTORC1 in developing rat brain is associated with decreased accumulation of HIF-1α (76), the main regulator of cellular adaptation to oxygen deprivation. Here we show that in primary astrocytes ischemic conditions, but not oxygen deprivation alone, are able to suppress mTORC1 activity and that inhibition via rapamycin treatment reduces HIF-1α levels. Yet, rapamycin treatment, although it moderately inhibits cap-dependent translation, does not affect protein synthesis rate and ATP steady state.

It was already described in cancer cells that rapamycin mediated mTORC1 inhibition evokes differential sensitivity towards the 4E-BP1 and S6K1 effector (175, 181, 182). For primary astrocytes we observed a similar discrepancy in susceptibility thresholds of mTORC1 signaling. Although ischemia completely dephosphorylated 4E-BP1 and S6K1, rapamycin treatment abolished S6K1

phosphorylation while it only partially affected the phospho-status of 4E-BP1 independently of time exposure. In bladder cancer cells rapamycin-driven S6K1 dephosphorylation triggered the loss of a negative feedback loop exerted by phosphorylated S6K1 onto IRS1, which eventually yield a pronounced activatory phosphorylation of AKT (175). In agreement with this, astrocytic AKT was activated by prolonged rapamycin treatment. Since other groups have shown that AKT can directly mediate 4E-BP1 phosphorylation in cancer and human embryonic kidney cells (136, 144), we speculate that this kinase might contribute to the sustained inactivation (phosphorylation) of the binding protein during rapamycin treatment. Although we have also shown analogous sensitivity of 4E-BP1 and S6K1 to rapamycin treatment in primary neurons, in these cells AKT activity was not affected by drug treatment, thus indicating cell specificity of the S6K1-feedback loop and its disruption by rapamycin.

It was recently shown in HeLa cells that inhibition of mRNA translation via 4E-BP1 dephosphorylation occurs during prolonged oxygen deprivation as the second stage of a biphasic process that, in the first instance, is regulated by very fast phosphorylation of eIF2α (83). However, our preliminary data in astrocytic cells indicate that phosphorylation of eIF2α occurs in parallel to 4E-BP1 dephosphorylation, similar to our observations in primary neurons (see section 7.4.4 and 7.5). These data suggest a differential kinetic of the mechanisms controlling mRNA translation in normal versus tumor cells and highlight distinct regulation of the mTORC1 signaling cascade in cells of different origin.

During stress conditions cancer cells effectively inhibit mTORC1 to foster 4E-BP1 hypo-phosphorylation, which is known to contribute to the inhibition of cap-dependent translation, the reduction of ATP consumption and to improve

survival of cells (47, 149). Similarly, in ischemic primary astrocytes hypo-phosphorylation of 4E-BP1 prevented the interaction of eIF4E with eIF4G within eIF4F complex as observed in HeLa cells (83). However, short-term inhibition of cap-translation during oxygen and glucose deprivation through dephosphorylated 4E-BP1 did not further inhibit overall protein synthesis compared to anoxia, where hyper-phosphorylated 4E-BP1 prevails. Extended period of oxygen deprivation caused an overall decrease of protein synthesis rate compared to shorter exposure, although 4E-BP1 phosphorylation did not change. Moreover, prolonged ischemia showed a trend to further reduce overall mRNA translation compared to anoxic condition. These results, suggest that 4E-BP1 dephosphorylation has no impact on global protein synthesis rate during shorter exposure to severe stress conditions while it still may inhibit translation of capped mRNAs during prolonged ischemia. Studies on the role of 4E-BP1 phosphorylation with respect to the regulation of global protein synthesis are controversial. While the state of 4E-BP1 phosphorylation does not seem to have an effect on overall mRNA translation in isolated rat hepatocytes (183), on the other hand, Connolly and colleagues reported that 4E-BP1 in breast cancer cells partially regulates global translation during hypoxia (176). Therefore, the impact of dephosphorylated 4E-BP1 on overall protein synthesis and ATP steady state appears to hinge on the background of the cells (normal vs transformed) and/or the severity and duration of stress. Clearly, this issue should be further investigated.

Inhibition of mRNA translation is part of a cellular strategy to coordinately decrease energy consumption along with the reduced ATP production of O_2-deprived cells in a controlled and fully reversible manner. The newly balanced

ATP steady state, termed hypometabolism, prevents lethal falls in cellular energy levels and is the single most protective and unifying feature of hypoxia tolerant tissues (47). Indeed, ATP levels were not affected by oxygen deprivation alone or rapamycin treatment indicating that the degree of protein synthesis rate reduction was adequate to preserve ATP levels. However, ischemia, particularly prolonged exposure, caused a massive depletion of cellular ATP while protein synthesis rate was maximally suppressed (to 5% of normoxic controls), thus revealing the inability of the cell to prevent energetic depletion under extreme circumstances. Since ATP production mainly occurs via oxidative phosphorylation within the inner membrane of the mitochondrion, we investigated generic enzymatic activities in mitochondria using the MTT assay in response to various degrees of stress. Although extra-mitochondrial redox reactions (i.e. cytosolic enzymes) have been suggested by some to function as major MTT reductase systems even in rat astroglia cells (184), the majority of publications report evidence for mitochondrial (succinate) dehydrogenases, in brain and other cells, to be responsible for the reduction of MTT-like tetrazolium substrates to the corresponding water-insoluble formazan product (185, 186). Contrary to the findings of the Takahashi study (184), we too observed that normoxic, hypoxic or anoxic cultures of astrocytes in glucose-deficient medium did not alter their MTT conversion, as one might expect if glycolytic redox reactions would account for the turnover of the tetrazolium compounds. Rather, glucose-deprived cells, relative to glucose proficient ones, displayed a moderate and O_2-independent decline in the conversion rate of MTT over time. Such cellular response would agree with the assumption that mitochondrial redox centers, whose electron transport is not (yet) limited by oxygen, are underlying

the reduction of tetrazolium dyes in astrocytes as well. However, this interpretation cannot explain the poor coupling between ATP steady state and MTT conversion data, especially at the 36h time point. While MTT conversion might well occur through mitochondrial enzymes, the fact that their activity does not seem to be linked to ATP production is a somewhat puzzling finding.

Despite these open questions, a recent study from Liu et al confirmed our results by demonstrating that in primary astrocytes ischemia induced ATP release (187) suggesting that this mechanism could also affect ATP levels and might only worsen energetic state of the cells.

Astrocytic responses to oxygen deprivation involve stabilization and accumulation of HIF-1α in response to very low tensions of oxygen (23). Here we show that rapamycin-dependent mTORC1 inhibition yields a weaker accumulation of HIF-1α during hypoxic or anoxic challenges in agreement with previous studies on cancer cells (188–190) and with our own results on neuronal cells (see section 7.4.7). Moreover, we observed an attenuated induction of HIF-1α after prolonged exposure to oxygen deprivation compared to shorter incubation times (36h versus 16h), which agrees well with previous results on the transient induction of HIF-1α (peak induction after 4 hrs; weakening induction after 12 hrs) in O_2-deprived human lung epithelial cells (72) as well as among different organs (191). These data, therefore, indicate that under our conditions mTORC1-mediated translation of HIF-1α contributes to the stabilization and accumulation of this subunit in hypoxic glia cells.

Although 4E-BP1 is the best known isoform of the 4E-BP family, 4E-BP2 is preferentially expressed in the brain (138, 139). We show that 4E-BP1 is the most abundant isoform in astrocytic cells, in agreement with previous data from

Roffé et al. (192) and contrary to our results on neuronal cells, where 4E-BP2 was preferentially expressed (see section 7.4.2). Moreover, contrary to 4E-BP1, astrocytic 4E-BP2 did not undergo detectable changes, highlighting 4E-BP1 as the isoform responsible for astrocytic responses under our conditions.

In conclusion, mTORC1 inhibition does not affect overall mRNA translation or ATP steady state during short-term ischemia. Despite complete oxygen and glucose deprivation–mediated dephosphorylation of 4E-BP1 and S6K1, global protein synthesis rates were not further inhibited and ATP depletion could not be rescued by rapamycin treatment. In addition, while inhibition of mTORC1 was persistent during prolonged ischemia, global proteins synthesis showed a trend to decrease, which anyway was inadequate to prevent the massive ATP depletion under such extreme stress conditions. The reduced HIF-1α accumulation in hypoxic/anoxic glia cells subjected to rapamycin suggests that mTORC1 inhibition may indirectly alter, if not restrict, the regulation of the transcriptional machinery, and through that, the adaptation of astrocytes to falling levels of O_2.

7.4 NEURONAL RESPONSE TO ACUTE (16 HOURS) AND PROLONGED (36 HOURS) OXYGEN DEPRIVATION

7.4.1 Rapamycin improves neuronal survival during prolonged oxygen deprivation

We investigated oxygen deprivation-mediated neuronal cytotoxicity by LDH assay (Fig. 1). Although 16 hours of mild oxygen deprivation (16h Hx, 1% O_2) hardly diminished cell viability, prolonged hypoxia (36h Hx) severely increased cytotoxicity to virtually 100% ($P<0.0001$). To investigate modulation of mTORC1/4E-BP1 signaling in neurons challenged with oxygen deprivation we inhibited mTORC1 directly via treatment with non-toxic doses of rapamycin. 10 nM of drug exposure moderately increased cell death to almost 12% ($P<0.01$), but only when cells were exposed to 16 hours near-anoxia (Ax, 0.2% O_2). Interestingly, however, rapamycin significantly reduced hypoxic cytotoxicity during prolonged (36h) exposure (Fig. 1B), in agreement with a recent study showing beneficial effect of the drug following oxygen and glucose deprivation (193). These data suggest that active mTORC1 signaling contributes to sustain survival of neurons during moderate or short-term oxygen scarcity, while inhibition of mTORC1 may be pivotal for protection from detrimental effects during prolonged hypoxia.

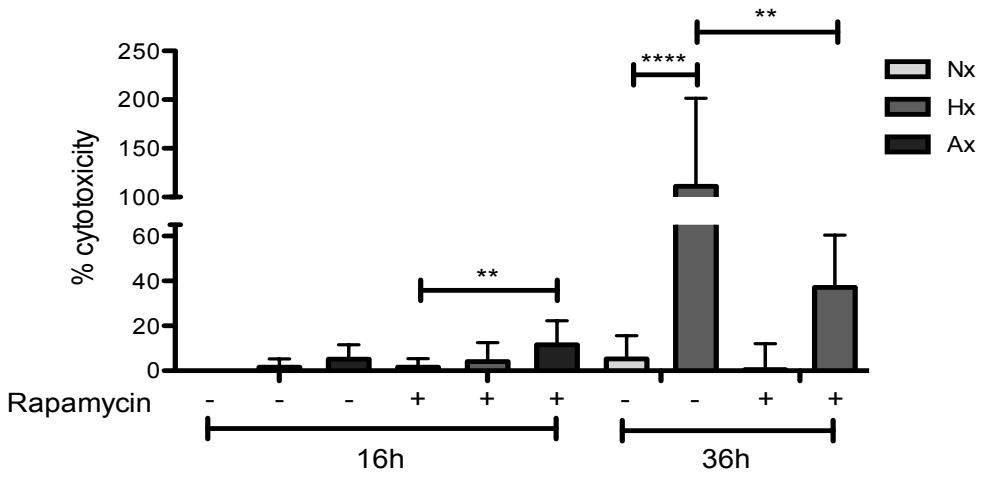

Fig. 1 Rapamycin improves neuronal survival during prolonged oxygen deprivation. Increased release of LDH indicates an increase in neuronal cell death under prolonged oxygen deprivation. After 36h exposure, there was a reduction of LDH release following 10 nM rapamycin treatment in line with a decrease in hypoxic cytotoxicity. Values are means ± SD (n=9). **p<0.01, ****p<0.0001

7.4.2 4E-BP2 is the most abundant species in neuronal cells but changes in regulation are not detectable

Three paralogous genes encode for the members of the eIF4E-binding protein family: 4E-BP1, 4E-BP2 and 4E-BP3 (132). While the role and regulation of 4E-BP3 still needs to be elucidated, 4E-BP2 was shown to predominantly regulate eIF4F in the brain (138). Hence, we examined 4E-BP2 versus 4E-BP1 abundance and regulation in neuronal response by immunoblot analysis (Fig. 2). In agreement with previous reports, 4E-BP2 was far more abundantly expressed than 4E-BP1 in primary neurons, yet failed to show at protein level any response to oxygen deprivation or 10 nM rapamycin exposure. These data demonstrate that 4E-BP2 is the isoform preferentially expressed in primary neurons.

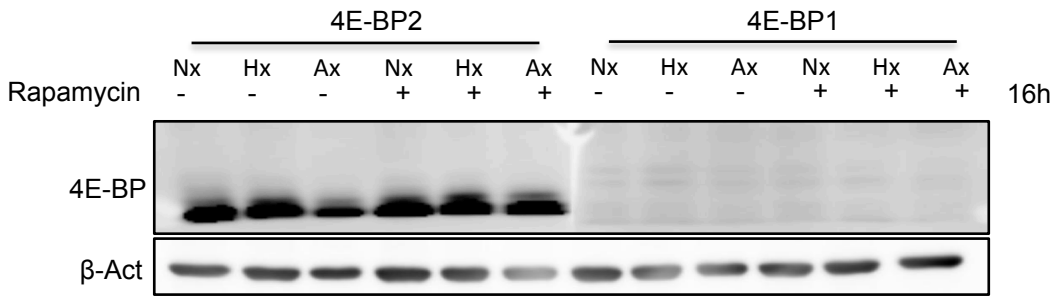

Fig. 2 4E-BP2 is the most abundant species in neuronal cells but changes in regulation are not detectable. Representative Western blot analysis of n=3 independent experiments shows that 4E-BP2 is more strongly expressed than 4E-BP1 but lacks at protein level any sensitivity to changes in O_2 supply or rapamycin application. β-actin was used as loading control.

7.4.3 Rapamycin treatment partially inhibits 4E-BP1 phosphorylation and completely dephosphorylates S6K1

Activity of the mTORC1 complex controls cap- and TOP-dependent translations via direct phosphorylation of 4E-BP1 and S6K1, respectively, a phosphorylation cascade that is inhibited by rapamycin. To study changes in 4E-BP1 phosphorylation, we performed immunoblot analysis (Fig. 3A). Unlike observations in astrocytes (see sections 7.1.1 and 7.2.1) 4E-BP1 was already partially hypo-phosphorylated (γ, β and α band) even under normoxic conditions and refractory in its phosphorylation status to 16 or 36 hours of hypoxic challenge. Surprisingly severe oxygen deprivation (Ax) and 10 nM rapamycin treatment only partially inhibited 4E-BP1 phosphorylation (γ, β and α band). In contrast, 16 hours of near-anoxia and rapamycin treatment decreased total levels of S6K1 to 70% ($P<0.001$ and $P<0.01$, respectively) of normoxia (Fig. 3A&B), although this negative impact on S6K1 levels was not seen in hypoxic exposure and prolonged (36h) drug treatment. S6K1 phosphorylation showed a trend to further decrease under near-anoxia (Fig. 3A&C) while rapamycin treatment completely abrogated it independently of time exposure and oxygen deprivation. These data indicate differential sensitivity of the phosphorylated status of 4E-BP1 and S6K1 to mTORC1 inhibition.

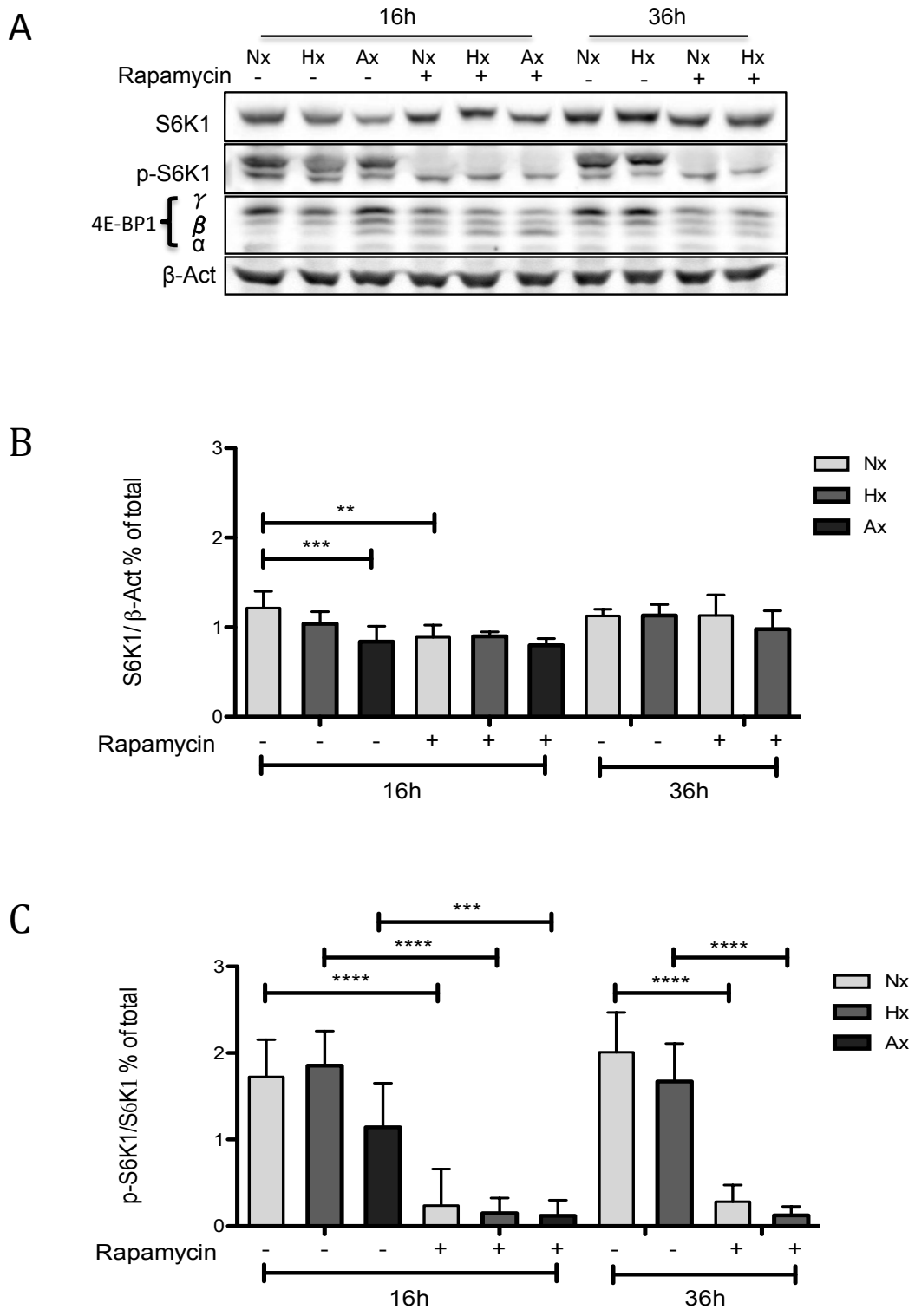

Fig. 3 Rapamycin treatment partially inhibits 4E-BP1 phosphorylation and completely dephosphorylates S6K1. A) Representative Western blot analysis (n=4 independent experiments) shows regulation of total and phosphorylated (p-) S6K1 and

phosphorylation states of 4E-BP1 (γ, β and α bands). β-actin was used as loading control. Protein levels were quantified by densitometry, graphs represent ratio of S6K1/β-actin B) and p-S6K1/S6K1 C). Values are means ± SD (n≥3). **p<0.01, ***p<0.001, ****p<0.0001

7.4.4 Rapamycin treatment does not affect AKT signaling and moderately increases eIF2α phosphorylation.

Our results in primary astrocytes showed that rapamycin treatment completely inhibited S6K1 phosphorylation although it only marginally affected 4E-BP1 (see sections 7.1.1 and 7.2.1). In glia cells drug treatment and S6K1 dephosphorylation yielded the loss of a negative feedback loop, thus activating AKT, which, kept 4E-BP1 in a partially hypo-phosphorylated state. In neurons total and phosphorylated AKT levels were non-responsive to oxygen deprivation or rapamycin treatment (Fig. 4A). Therefore, AKT activity is not modulated under the chosen conditions.

eIF2α is another player of the cap-dependent translation, important for the assembly of the 43S pre-initiation complex (194). While total and phosphorylated levels of eIF2α did not change during either oxygen deprivation or rapamycin treatment alone (Fig. 4A&B), the phosphorylation of eIF2α significantly increased by almost 38% ($P<0.05$) upon exposure to combined near-anoxic and drug-presenting cultivation of primary neurons. Although eIF2α phosphorylation is not altered through any of our oxygen deprivation schemes, phosphorylation of this initiation factor is elevated in response to rapamycin treatment during severe stress conditions.

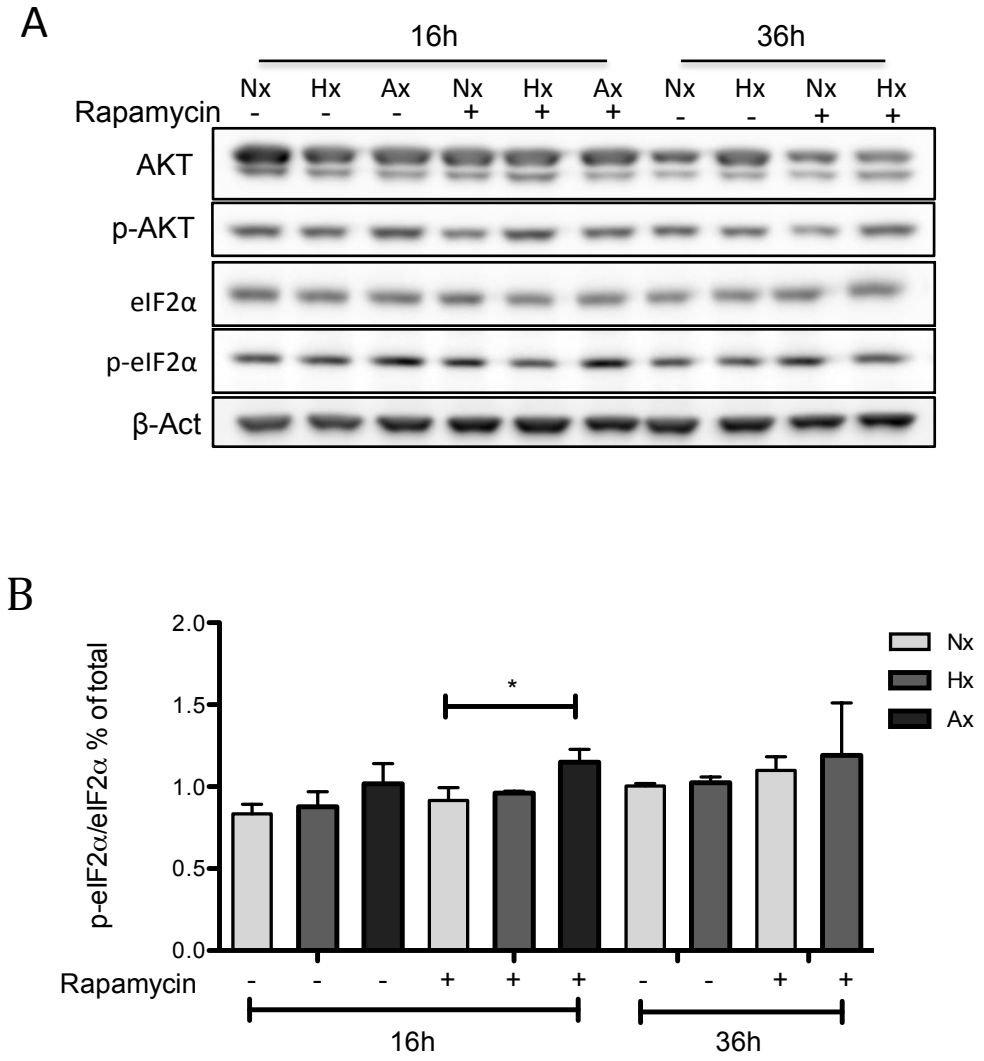

Fig. 4 Rapamycin treatment does not affect AKT regulation but moderately increases eIF2α phosphorylation. A) Representative Western blot analysis (n=3 independent experiments) of total and phosphorylated (p-) AKT and eIF2α. β-actin was used as loading control. B) Proteins were quantified by densitometry and graph shows the ratio of p-eIF2α/eIF2α. Values are means ± SD (n=3). *p<0.05

7.4.5 Rapamycin-dependent 4E-BP1 hypo-phosphorylation does not affect protein synthesis rate or cap-dependent translation

Since mTORC1 regulation of 4E-BP1 and S6K1 pathway ultimately modulates global mRNA translation, we analyzed overall protein synthesis rate under our experimental conditions (Fig. 5A). For a better understanding of the mechanisms, we included 36 hours near-anoxia exposures to challenge the cells with a more severe stress. Compared to normoxic controls, hypoxia surprisingly did not attenuate global mRNA translation, while 16 hours severe oxygen deprivation (Ax) decreased it to 60.4%±18.8 ($P<0.01$). As expected, prolonged Ax exposure (36 hours) further reduced it to 48.2%±22.1 ($P<0.01$) of the control (Fig. 6A). Moreover, 10 nM rapamycin treatment did not alter the rate of protein synthesis beyond the degree of inhibition by O_2-deficiency. These data suggest that incomplete 4E-BP1 hypo-phosphorylation, as demonstrated for a 16h Ax or rapamycin challenge (Fig. 3A), is not sufficient to modify total mRNA translation in primary neurons. Since 4E-BP1 is usually regarded as direct modulator of cap-dependent translation, we investigated its ability to interact with eIF4E using an *in vitro* cap-capture assay (Fig. 6B). 4E-BP1 phosphorylation of Thr46 triggers the phosphorylation cascade that results in its release from eIF4E (137). Hence we used non-phospho-4E-BP1 (Thr46) antibody to specifically detect the active species that bind eIF4E. Interestingly, 4E-BP1 interaction with eIF4E occurred even under non-stress conditions (36hNx), while 16 and 36 hours hypoxia actually decreased the association (Fig. 6B; lanes 36hNx versus 16hHx, 36hHx and 36hAx). Moreover, 16 hours near-anoxia and rapamycin exposure did not affect the interaction of 4E-BP1 with eIF4E compared to the normoxic control

(Fig. 6B; lanes 36hNx versus 16hAx and 36hNx+Rapamycin). Since near-anoxic condition and drug treatment partially hypo-phosphorylated 4E-BP1 (Fig. 2A; α band), these data indicate that 4E-BP1/eIF4E binding is not directly correlated with levels of 4E-BP1 phosphorylation. In neurons, the rate of cap-dependent translation appears to primarily depend on the assembly of eIF4E with eIF4G to initiate the eIF4F complex. This interaction, although refractory to 16 hours hypoxic or rapamycin exposure (Fig. 6B; lanes 36hNx versus 16hHx and 16hNx+Rapamycin), was clearly and sensitively antagonized by 16 hours anoxia and 36 hours of hypoxic or anoxic oxygen deprivation. These results indicate that primary neurons facing chronic hypoxic or anoxic stresses regulate the initiation of cap-dependent protein synthesis most probably via the binding of eIF4E with eIF4G, and rather not via eIF4E scavenging by 4E-BP1.

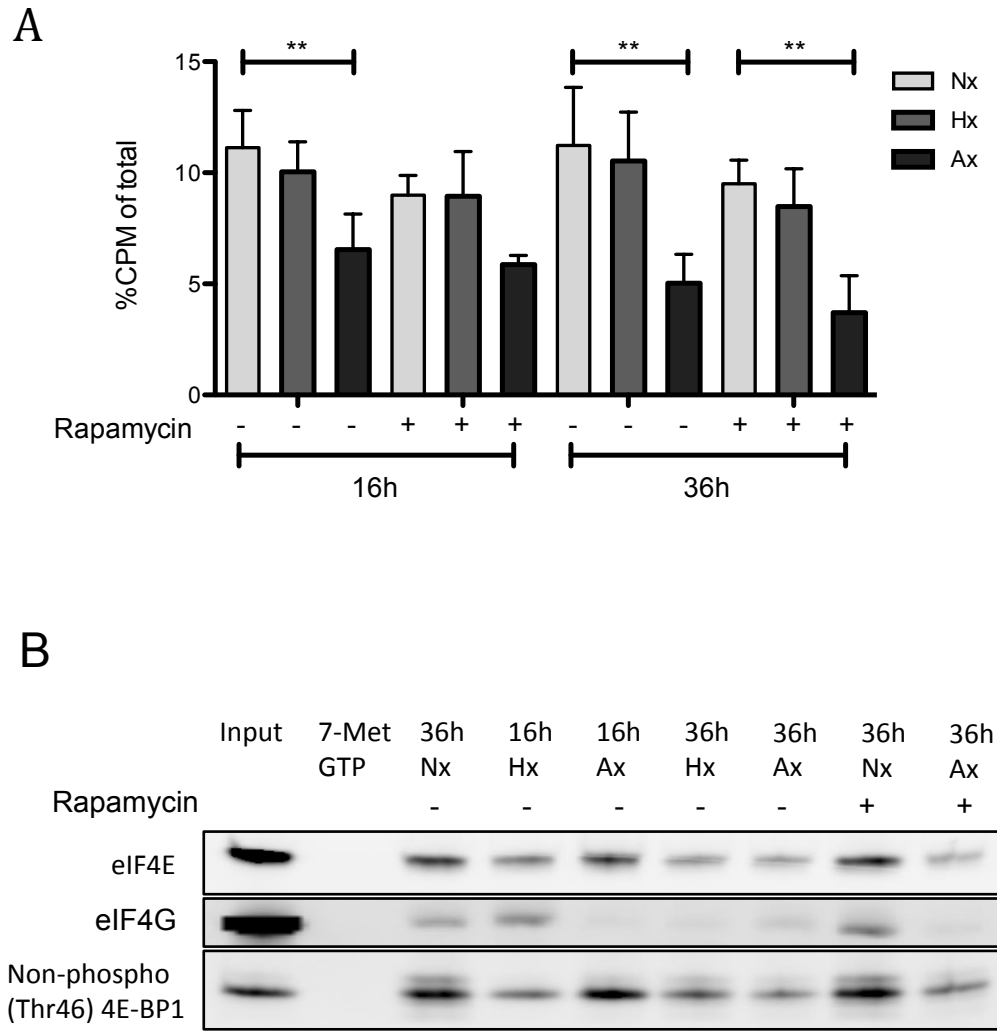

Fig. 5 Rapamycin-dependent 4E-BP1 hypo-phosphorylation does not affect protein synthesis rate and cap-dependent translation. A) Overall protein synthesis rate decreases under severe oxygen deprivation but is not additionally inhibited by drug treatment. Values are means ± SD (n=3). **p<0.01 B) Representative *in vitro* cap-affinity assay (n=2 independent assays) shows diminished binding of eIF4E to eIF4G following oxygen deprivation, insensitivity of the eIF4E/4G complex formation to rapamycin and a strong competitive binding of dephosphorylated 4E-BP1 to eIF4E already under normoxic conditions. Whole cell extract (Input) was used as positive control for protein extraction and 7-Met GTP as negative control.

7.4.6 Rapamycin treatment does not alter ATP levels or mitochondrial activity

We analyzed steady state levels of cellular energy by ATP bioluminescence assay, to assess if dwindling protein synthesis rates seen in anoxic neurons (Fig. 6A) might aid in steading ATP levels during periods of O_2 scarcity. 16 hours hypoxic exposure and rapamycin treatment hardly affected the ATP steady state, while anoxic conditions reduced ATP to 62.2%±18.7 ($P<0.01$) of the control (Fig. 6A). Unexpectedly, during prolonged oxygen deprivation and drug treatment ATP levels were more or less stable (Fig. 6A). We currently cannot explain why 36 hours of rapamycin-treated normoxic neuronal cells displayed a, though variable, measurable increase in ATP levels.

Since ATP is mainly produced through oxidative phosphorylation in mitochondria, we investigated neuronal mitochondrial activity by using MTT assays. While 16 h hypoxia was, surprisingly enough, insufficient to trigger a significant fall in oxidative enzymatic activities, 16h anoxia reduced, relative to control values, MTT turnover by 30% ($P<0.01$). Beyond this O_2-dependent MTT activity, rapamycin application did not add any further impact on the substrate turnover by mitochondria (Fig. 6B). However, prolonged hypoxic and anoxic exposure inhibited mitochondrial activity by 30% ($P<0.01$) and 55% ($P<0.0001$), respectively (Fig. 6B).

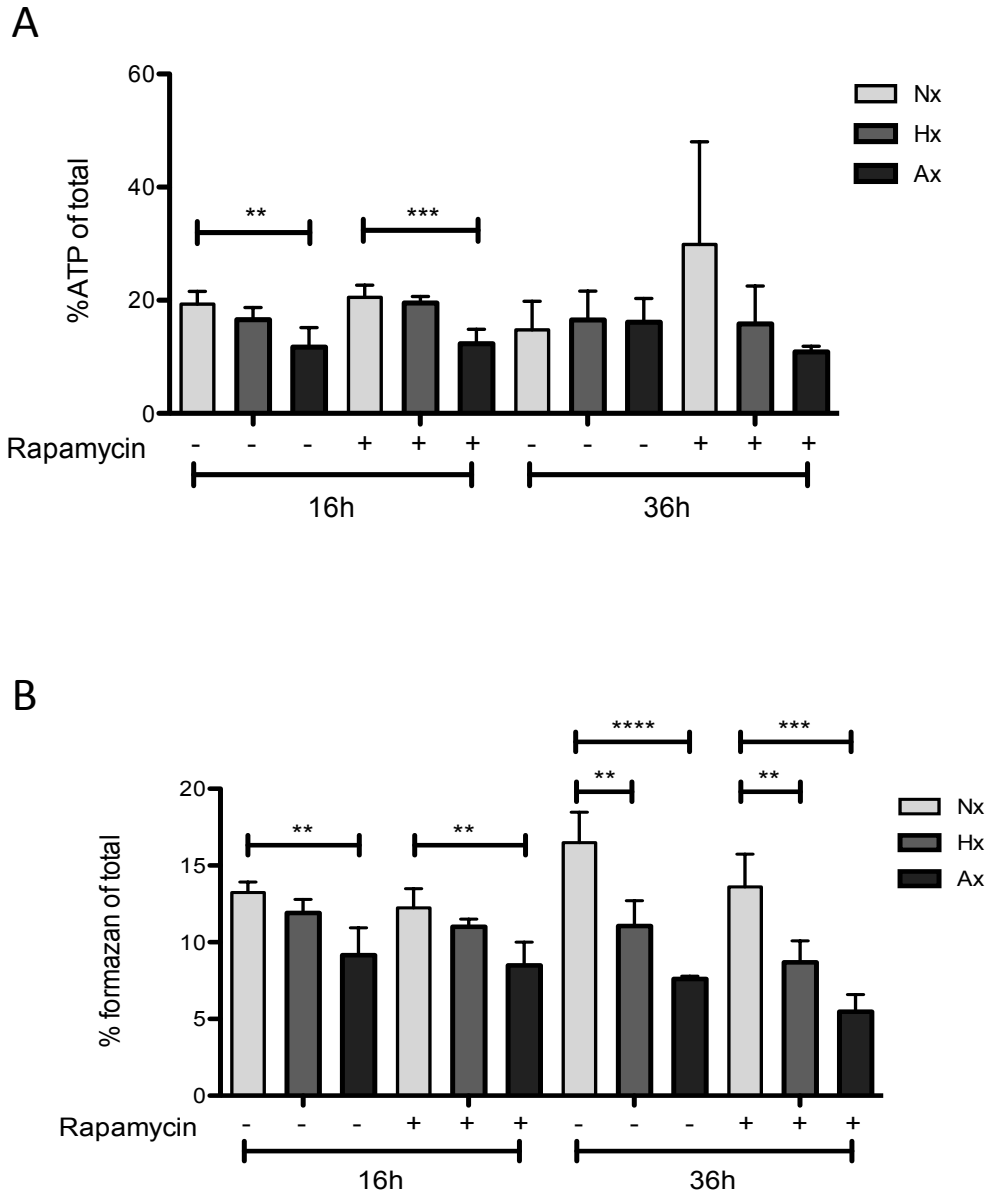

Fig. 6 Energy levels and mitochondrial activity decrease during severe oxygen deprivation but are unaffected by rapamycin. A) ATP steady state levels are significantly reduced by 16 hours but not affected by 36 hours of severe oxygen deprivation. Values are means ± SD (16h n=4; 36h Nx and Hx n=3; 36h Ax n=2). B) MTT levels indicated graded decline of oxidative activity when neurons were subjected to 16 hours and 36h of normoxic, hypoxic and anoxic exposure. Cells were also exposed to 10 nM rapamycin. Values are means ± SD (n=3). **p<0.01, ***p<0.001, ****p<0.0001

7.4.7 Rapamycin decreases HIF-1α stabilization during prolonged mild oxygen deprivation

Since recent studies indicate a direct control of HIF-1α expression by the mTORC1/4E-BP1 pathway in transformed cells (188) we hypothesized that rapamycin treatment may similarly modulate neuronal HIF-1α . As expected, Hif-1α was detectably stabilized during mild and severe oxygen deprivation (Fig. 7). Rapamycin treatment reduced HIF-1α levels under prolonged (36 hours) hypoxia (Fig. 7). These data suggest that in primary neuronal cells HIF-1α accumulation may positively correlates with mTORC1 signaling during extended oxygen deprivation.

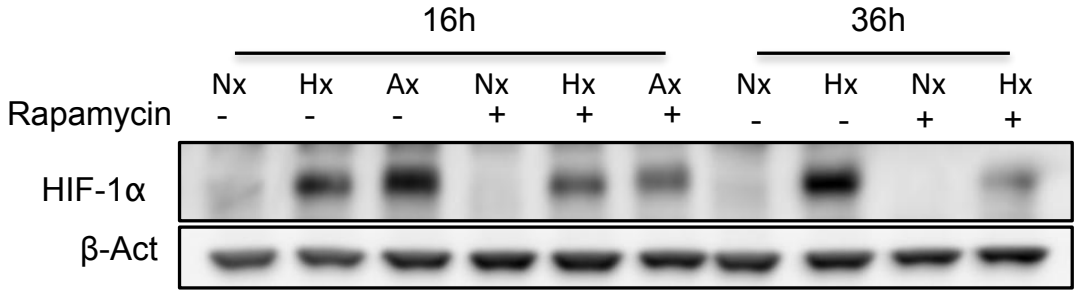

Fig. 7 Rapamycin decreases Hif-1α stabilization during prolonged mild oxygen deprivation. Representative Western blot analysis (n =6 independent experiments) shows stabilization of HIF-1α following oxygen deprivation. Exposure with 10 nM rapamycin decreased HIF-1α levels, particularly during prolonged hypoxia. β-actin was used as loading control.

7.5 DISCUSSION ON NEURONAL RESULTS

Oxygen and/or substrate deprivation disturbs homeostasis of the brain and results in deleterious effects (8). Due to the brains high energy consumption and strict dependence on oxygen and glucose as biochemical fuel (1), hypoxic or ischemic insults have long been known to trigger neuronal cell death within minutes (195, 196). More recently, several reports emphasized the fact that neurons are more sensitive to hypoxic insults than other cells (178, 197). Yet, the mechanisms behind this exceptional sensitivity of neuronal cells still need to be fully elucidated. A number of *in vitro* and *in vivo* studies have shown that mTORC1 signaling plays an important role in neuronal response to CNS injury (172, 173, 198, 199) and that its activity correlates with HIF-1α stabilization in developing rat brains (76). Our results are in agreement with others, showing that mTORC1 inhibition via rapamycin treatment improves neuronal survival during oxygen deprivation and decreases HIF-1α stabilization.

However, as long as glucose is not limiting, a recent study found neurons to be surprisingly resistant to hypoxic injury even for prolonged periods (200). In line with that study, we also observed cytotoxicity to only increase once neuronal cells were exposed to prolonged hypoxia. Moreover, similar to previous data in an *in vitro* model of stroke (193), rapamycin treatment rescued neuronal survival, suggesting that sustained activation of mTORC1 pathway in neurons during hypoxia partially mediates neuronal death.

mTORC1 is known to confer its signaling onto protein synthesis, and through that cell viability, via the phosphorylation targets S6K1 and 4E-BP1. Although 4E-BP1 is the most studied isoform of the 4E-BP family, 4E-BP2 was shown to be

preferentially expressed in the brain (138, 139). In agreement with these data, 4E-BP2 protein was also more abundant than 4E-BP1 in primary neurons. *In vivo* studies demonstrated the critical role of 4E-BP2 in synaptic plasticity and memory (139) and its association with autistic-like behavior (140), thus suggesting a major role of this protein in modulating brain activities. Moreover, a recent report indicated a correlation between increased association of 4E-BP2 to eIF4E and delayed neuronal death during ischemia reperfusion in the hippocampus (201). We could not detect changes of 4E-BP2 protein in hypoxic neurons and thus suggest that the modulation seen in the study from Ayus et al. could be either due to additional substrate deprivation during ischemic insult or caused by reperfusion-triggered effects (e.g. generation of reactive oxygen species, ROS). Therefore it may be possible that in primary neurons the two binding proteins are differentially regulated depending on the stimuli to which cells respond. Moreover, Grolleau et al showed that granulocytic differentiation causes a switch from 4E-BP1 to 4E-BP2 isoform and suggested the existence of specific genes whose translation may be regulated by one or the other binding protein (202). Synapses of *in vitro* neurons increase with culture time, showing morphological maturation from 7 DIV to 20 DIV (203, 204). Therefore, it is possible that also in primary neurons 4E-BP1 and 4E-BP2 occur at different *in vitro* differentiation stages. However, we require more studies for a better elucidation of the mechanisms undergoing the regulation of the two cap-dependent translation regulators.

It is well known that hypo-phosphorylation of 4E-BP1, triggered by the inactivation of mTORC1 during stressful environmental conditions, inhibits the initiation of cap-dependent protein synthesis (205). However, neurons showed

elevated basal levels of hypo-phosphorylated 4E-BP1 already during normal circumstances. It is tempting to speculate that these cells slow down mRNA translation initiation because of the reduced necessity of non-dividing cells to produce proteins. This hypothesis was further supported by eIF2α phosphorylation data. Indeed, together with 4E-BP1, eIF2α is known to be an important player in cap-dependent mRNA mechanisms. Phosphorylation of eIF2α inhibits the assembly of 43S pre-initiation complex, thus blocking protein synthesis in cancer (206) and normal cells (207) exposed to oxygen deprivation. However, in neuronal cells eIF2α was already phosphorylated during normoxic condition. Indeed reduced cap-dependent protein synthesis levels already under normal circumstances is in agreement with the dogma that post-mitotic cells require lower rate of protein synthesis compared to rapidly growing cells (208). Moreover, since 4E-BP1 hypo-phosphorylation only partially increased during anoxia and eIF2α regulation was not affected, it is probable that in neurons subjected to our conditions these effectors of cap-dependent translation are not as important as in cancer cells.

mTORC1 is critically involved in tuning cellular resistance to environmental changes due to its role in the cap- and TOP- translational regulation. mTORC1 inhibition via rapamycin treatment differentially affected 4E-BP1 and S6K1 regulation in various cancer cells and myoblasts (175, 181, 182). A recent *in vivo* report showed that reduction in the abundance of total and phosphorylated myocardial S6K1 correlated with rapamycin administration (209). Similarly, in primary neurons rapamycin treatment reduced total levels and completely inhibited S6K1 phosphorylation but only moderately reduced 4E-BP1 phosphorylation. We have shown comparable response also in primary

astrocytes and suggested a rapamycin-driven AKT activation mechanism to sustain 4E-BP1 phosphorylation (see section 7.3). In agreement, previous studies on cancer cells demonstrated that prolonged rapamycin treatment caused the loss of the S6K1/IRS1 negative feedback (210), which, in turn, yields AKT activation (175) and 4E-BP1 phosphorylation (136, 144). However, neuronal AKT activity was not raised by drug treatment, thus suggesting that other kinases may be responsible for the sustained partial phosphorylation of neuronal 4E-BP1 during rapamycin exposure. Although 4E-BP1 phosphorylation ultimately releases eIF4E, hence, resulting in active cap-dependent mRNA translation, its binding with eIF4E did not directly correlate with its hypo-phosphorylation state. Our data suggest that neuronal inhibition of cap-dependent translation during oxygen deprivation is probably more dependent on decreased eIF4E/eIF4G binding rather than sequestration of eIF4E by 4E-BP1. However, since in the last decade other inhibitory eIF4E partners have been identified (211), other binding proteins (e.g. 4E-BP2, masked, Neuroguidin, Cup) may regulate cap-dependent translation under our conditions. Nevertheless, so far due to lack of time we could not investigate alternative mechanisms. Although regulation of cap-dependent protein synthesis is believed to be mainly regulated by inhibitory proteins (126), cleavage of eIF4G or shuttling of eIF4E (by eIF4E-transporter; eIF4E-T) was also shown to modulate mRNA translation inhibition. Cells infected by poliovirus (212) or calcivirus (213), and cancer cells undergoing apoptosis (214, 215), shut-off protein synthesis via eIF4G inactivation. Since it was previously shown that hypoxia induces apoptotic neuronal death (216), it appears probable that under our conditions cleavage of eIF4G plays the major role in cap-dependent mRNA translation inhibition. In

addition, in HeLa cells interaction of eIF4E with eIF4E-T triggers the translocation of the complex to the nucleus (217), a mechanism that, together with 4E-BP1 hypo-phosphorylation, have been shown to inhibit cap-dependent translation in anoxic HeLa cells (83). In this regard, mores studies are required to address the role of eIF4E-T under our conditions. Nevertheless, dissociation of eIF4E and eIF4G did not always diminish overall protein synthesis rate. Indeed, eIF4F disruption and decreased global mRNA translation correlated only during near anoxic condition (16h and 36h Ax). These results agree with our data on primary astrocytes where inhibition of eIF4F complex via binding of 4E-BP1 to eIF4E also failed to reduce global protein synthesis (see section 7.1.2). This is in contrast to observations in breast cancer (176) and PC12 cells (205) where modulation of the 4E-BP1 phosphorylation state was tightly associated with changes in global translation during oxygen deprivation. We thus conclude that the comparatively low protein and RNA synthesis rate of slow-growing and post-mitotic cells (208) probably renders the assembly and disassembly of the eIF4F far less responsive to environmental signals or pharmaceutical compounds.

Inhibition of mRNA translation is a mechanism activated by stressed cells to steady ATP turnover at times when little ATP can be produced (47). Indeed, ATP levels showed no sign of reduction, or even depletion, during 16 and 36 hours of hypoxia, in correlation with ongoing overall protein synthesis. Our studies, in agreement with previous reports showing high resistance of immature neurons to reductions of ATP levels during oxygen deprivation (218), showed falling energy levels (relative to controls by ~40%) only during severe stress conditions. At 16 hours anoxia, energy savings from moderately decreased protein synthesis rates (down to ~60% of controls) were obviously inadequate

to balance the decreased ATP production of the cells, thus causing a drop in ATP concentration. At 36 hours anoxia, however, the equally suppressed protein synthesis was surprisingly able to stabilize ATP levels at control niveau, despite the dramatically increased incidence of cell death as measured by LDH release assays. According to these data, a steady ATP turnover does, in neurons at least, not seem to prevent or delay high cell mortality. Currently, we can not explain the disparity of reduced cell survival and protein synthesis rate versus maintained neuronal energy levels. Nevertheless, since the mitochondrion is the main producer of ATP in eukaryotic cell types, general evaluation of its function could help to clarify such a response. Under our conditions, oxygen deprivation reduced MTT levels in a graded fashion (i.e. Ax > Hx > Nx and 36h (O_2 deprivation) > 16h (O_2 deprivation), thus illustrating the progressive mitochondrial impairment in O_2-limited nerve cells. Reduction of proteins synthesis rate, ATP and MTT levels were synchronized as expected at shorter time points. Maintained ATP levels during 36 hours challenges, however, can only result from the apparent coordination in the reduced energy production (mitochondrial activity) and consumption (e.g. protein synthesis rates), hence reflecting the completely unexpected prevention of any measurable energy loss in chronically hypoxic or anoxic immature neurons. The reason for this discrepancy at the late time, and particularly the finding that this excellent re-programming of ATP production/consumption cannot prevent a major kill of cells, remains entirely unclear but would seem most likely to be a technical problem. However an unknown biological response cannot be excluded and should be further investigated.

Notably, HIF-1α largely contributes to the mechanisms activated by neuronal cells during hypoxia (64, 219). In cancer cells, rapamycin-driven mTORC1 inhibition correlated with decreased HIF-1α stabilization due to the impaired translation of the subunit (188–190). For neurons, we also show that inhibition of mTORC1 via rapamycin decreases HIF-1α stabilization when cells are exposed to hypoxic stress. Thus, some parallels do exist between mitotic and post-mitotic cells in response to mTORC1 inhibition during O_2 deprivation.

Altogether we can conclude that mTORC1 pathway is involved in the mechanisms of cell death activated in neuronal cells during prolonged oxygen deprivation. However, since 4E-BP1 phosphorylation state and eIF4E binding capacity are hardly affected by varying degrees of O_2-deprivation, it seems unlikely that this binding protein isoform plays a major role in adjusting protein synthesis to external stimuli. Perhaps, other factors may be more relevant in this regard for neurons (i.e. 4E-BP2, eIF4E-T).

It also seems that 4E-BP1 hypo-phosphorylation has little effect on protein synthesis rate or ATP levels reduction during near-anoxia. Indeed, although rapamycin and near-anoxic exposure caused a similar decrease of 4E-BP1 phosphorylation, during drug treatment no additional effects were detected on global mRNA translation and energy levels. Also S6K1 activity does not seem to play a fundamental role in such mechanisms under our conditions. Indeed, we could not detect any change on protein synthesis rate or cellular energy during drug treatment, although the complete inhibition of S6K1 phosphorylation. On the other hand, exposure to rapamycin rescued neuronal viability and reduced HIF-1α accumulation during long-term challenges, thus suggesting a role of this transcriptional factor as potential player in promoting cell death mechanisms in

neurons subjected to prolonged hypoxia. In agreement, recent *in vivo* studies revealed the association of HIF-1α and neuronal apoptosis after brain injury and indicated p53 and BNIP3 (HIF target genes) as probable regulators of such mechanism (220, 221). However, due to the broad range of genes regulated by HIF-1, it can not be excluded that other proteins may be involved in the neuronal cell death mechanism under our conditions.

8 Conclusions

The main focus of this thesis work was to understand how mechanisms involved in astrocyte and neuronal responses to oxygen deprivation trigger their differential sensitivity and might underlie the enhanced astrocytic resistance to hypoxia or anoxia. Since mTORC1 pathway has been shown to be a central regulator of hypoxic responses, we investigated the role of this signaling cascade, and particularly of the mTORC1 effector 4E-BP1, in neuronal and astrocytic cells after oxygen deprivation.

Even though the original question directed at understanding the mechanisms behind the differential stress susceptibility remains open, a number of additional issues also arise from this work. In primary brain cells, the role of mTORC1 and its effectors does not seem to fit with the current knowledge that is mainly gained from cells with cancer background. Notably, in cancer cells alterations of many signaling cascades, including mTORC1 pathway, are required to increase protein synthesis and proliferation rates. Along similar lines, putative alternative mTORC1 functions, while occurring at minimal activity or in a latent state in neoplastic backgrounds, might actually play more important roles in normal growing or post-mitotic primary cells. Current evidence proposes hypo-phosphorylated 4E-BP1 to act as translational brake as well as being a regulator of mitosis. We now show for primary astrocytes that 4E-BP1 functions as a delimiting factor of cap-dependent initiation of translation when *hypo*-phosphorylated during ischemia (*in vitro*: anoxia and glucose withdrawal). Beyond its canonical role, however, we provide evidence that *hyper*-phosphorylated 4E-BP1 in oxygenated glia cells stimulates protein synthesis rate

and cell cycle progression, rather than occupying the widely believed role of an inactive bystander. The mechanisms and partner proteins for this non-canonical behavior have yet to be uncovered. Therefore, it would be interesting to investigate the role of hyper-phosphorylated 4E-BP1 in other rapidly dividing cells as well as post-mitotic neuronal cells. Moreover, since neuronal 4E-BP1 is already hypo-phosphorylated under normal conditions (i.e. normoxia), stress-induced additional hypo-phosphorylation is minimal, and it seems to play a marginal role in controlling cap-translation during O_2 scarcity. Thus, it would not be unrealistic to perceive astrocytic 4E-BP1 as a highly selective factor that controls only a small group of mRNAs, maybe those involved in synaptic plasticity or neurotransmission of nerve cells.

The question of which mechanisms drive differential sensitivity of neurons and astrocytes still has no definitive answer but remains crucial. A better understanding of these processes would be beneficial to extend our knowledge of mechanisms activated during normal conditions as well as those that define outcome after injury in brain cells. Undoubtedly, such insight would potentially identify novel therapeutic targets for pharmacological treatment of hypoxic dependent CNS injury.

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10 Manuscript

All the experiments present in the following manuscript, submitted to MCB (Molecular and Cellular Biology) journal, were performed by the thesis author (Daniela Nizzari).

Phosphorylated 4E-BP1 stimulates cell cycle and overall protein synthesis in non-stressed primary astrocytes

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ABSTRACT

Protein synthesis and cell division can be compromised in CNS pathologies including stroke, an injury leading to gliosis via astrocyte activation and hypertrophy, and malignant gliomas, often characterized by pronounced deregulation of mRNA translation and cell proliferation. Eukaryotic translation initiation factor 4E (eIF4E) triggers the binding of eIF4F complex to the 5' cap structure of mRNAs and initiates transcript recruitment to ribosomes. Hypo-phosphorylated eIF4E binding protein 1 (4E-BP1) sequestrates eIF4E, thus inhibiting translational initiation. In contrast, hyper-phosphorylation of 4E-BP1 via mammalian target of rapamycin complex 1 (mTORC1) inactivates the protein. As most of our knowledge on 4E-BP1 come from pathological contexts, we investigated modulation and function of 4E-BP1 in normal or oxygen and/or glucose deprived primary astrocytes exposed to mTORC1 inhibitors or small interfering RNA-mediated gene silencing. During oxygen and glucose deprivation, 4E-BP1 dephosphorylation inhibited cap-dependent protein synthesis but did not affect global translation. Interestingly, knockdown of 4E-BP1 reduced cell proliferation and overall protein synthesis in normoxic astrocytes, i.e. when 4E-BP1 is hyper-phosphorylated, while no effects were observed during oxygen deprivation. Our *in vitro* experiments confirm hypo-phosphorylated 4E-BP1 as translational brake in severely stressed astrocytes but also reveal hyper-phosphorylated 4E-BP1 as stimulator of cell growth and proliferation in oxygenated glia.

INTRODUCTION

The regulation of translation influences many cellular activities including cell growth, proliferation and responses to environmental stimuli. Although many pathways modulate protein synthesis through various eukaryotic initiation factors (eIFs), cap-dependent translation is the synthesis mechanism for the majority of polypeptides (1–3). Most mRNAs possess a cap structure, m^7GpppN (where m: methyl group; G: guanine base; ppp: triphosphate group; N: any nucleotide), at the 5' terminus to facilitate recruitment of the eIF4F complex to ribosomal subunits for the initiation of cap-dependent translation (3, 4). Regulation of the initiation of protein synthesis via binding to the 5' cap of mRNAs is the most controlled phase of translation, and is considered to be the rate-limiting step of the process (5). The complex eIF4F is composed of three eIFs: the cap-binding factor (eIF4E), the scaffolding protein 4G1 or 4G2 (eIF4G1/2) and the helicase 4A (eIF4A) (3). eIF4E-binding proteins 1-3 (4E-BPs) modulate the assembly of eIF4F and inhibit cap-dependent translation via their interaction and sequestration of cap-binding eIF4E subunit. Although the three members of the 4E-BP family show similar inhibitory activity (6), 4E-BP1 has been most characterized. In its hypo-phosphorylated state 4E-BP1 directly binds eIF4E, thus competing with the eIF4E/4G association within the eIF4F complex and impeding activation/initiation of cap-dependent translation (7). In contrast, phosphorylation on serine/threonine side chains of 4E-BP1 leads to its dissociation from eIF4E, allowing eIF4F complex assembly and cap-dependent protein synthesis initiation. mTORC1, a protein complex composed of mammalian target of rapamycin (mTOR) kinase, GβL and regulatory-associated

protein of mTOR (raptor), phosphorylates 4E-BP1 and also ribosomal protein S6 kinase 1 (S6K1). Contrary to the inactivation of the 4E binding protein, mTORC-driven phosphorylation of S6K1 functions as stimulatory signal, leading to the activation of the 5′-terminal oligopyrimidine tract (TOP) -dependent translation of ribosomal components and translation elongation factors. Incoming cytokine, hormone, amino acid and growth factor signals activate mTORC1, thereby positively stimulating mRNA translation via phosphorylation of its downstream substrates 4E-BP1 and S6K1.

Stress conditions such as hypoxia and nutrient deprivation inhibit the mTORC1 pathway causing 4E-BP1 dephosphorylation that in turn suppresses mRNA translation and fosters energy conservation and cell survival. Such translational regulation has been shown to play a fundamental role in cancer development, growth and therapeutic resistance (8, 9). Activation of cap-dependent protein synthesis by hyper-phosphorylation of 4E-BP1 is associated with increased cancer progression and decreased survival in astrocytoma (10), while reduced expression was found to increase resistance to drug treatment in glioma cells (11). However, loss-of-function of 4E-BP1 in xenografts of U87 glioblastoma cells resulted in marked attenuation of hypoxia tolerance and increasing radiosensitivity (8). Indeed, during stress conditions tumors inhibit cap-dependent translation via 4E-BP1 hypo-phosphorylation, thus reducing energy consumption and improving cell survival (8, 12). A similar mechanism was activated during oxygen deprivation in isolated rat hepatocytes (13).

So far, knowledge of 4E-BP1 function has been mainly derived from cancer cells and primarily focused on the hypo-phoshorylated form as the major antagonist of cap-dependent translation. However, the factor was recently shown to

regulate cell cycle progression in embryonic fibroblasts (14), HeLa (15) and HepG2 cells (16). Clearly the potential function of the as yet unstudied hyper-phosphorylated 4E-BP1 and the role of this regulator in normal untransformed cells need to be investigated. It is well known that astrocytes are highly stress-resistant brain cells (17) and play a neuroprotective role during brain disease (18). The mTORC1 pathway was recently shown to be involved in the physiological response of astrocytes to injuries of the CNS (19). Since alterations in 4E-BP1 total levels and phosphorylation state are associated with astrocytic cancer development and progression (8–10), we hypothesize that in its hyper-phosphorylated state 4E-BP1 may play a role in regulating cell growth and proliferation also in normal untransformed glia cells.

MATERIALS AND METHODS

Chemicals and reagents

Cell culture media and reagents were purchased from Gibco® (Life Technologies, Zug, Switzerland). Rapamycin was obtained from Sigma-Aldrich (St. Louis, MO) and NVP-BEZ235 hydrocloride (NVP) from Chemdea (Ridgewood, NJ). Oligofectamine™ Reagent was ordered from Invitrogen (Carlsbad, CA) and ON_TARGETplus SMARTpool targeting 4E-BP1 (L-096117-01), S6K1 (L-099323-02) and non-targeting pool (D-001810-10) from Thermo Scientific (Waltham, MA). Protease inhibitor cocktail Set III was purchased from Calbiochem (Merck, Darmstadt, Germany). Pierce BCA Protein Assay was obtained from Thermo Fisher Scientific Inc. (Rockford, USA). Antibodies directed against 4E-BP1, non-phospho-4E-BP1(Thr46), S6K1, phospho-S6K1 (Thr389), AKT, phospho-AKT (Ser473), eIF2α, phospho- eIF2α, eIF4G were from Cell Signaling (Danvers, MA),

eIF4E from BD Bioscience (San Jose, CA) and β-actin antibody from Sigma-Aldrich (Buchs, Switzerland). Secondary antibodies for Western blotting were obtained from Jackson ImmunoResearch (Suffolk, UK). 5-bromo-2'-deoxyuridine (BrdU) incorporation kit was from Roche (Mannheim, GE), m^7GTP-sepharose 4B from GE Healthcare (Glattbrugg, Switzerland) and 7-methylguanosine 5'-triphosphate from Sigma-Aldrich (Buchs, Switzerland). [^{35}S] methionine/cysteine was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO).

Primary culture of astrocytes

Primary astrocytes were isolated from newborn Wistar rat pups as previously described (17, 20). Briefly neonatal rats were decapitated, cerebral cortex and meninges removed, and cortices minced and placed in ice-cold buffer (Krebs solution; 120mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, and 0.6 g/l BSA). Tissue was then homogenized and digested with 7.5 mg trypsin for 15 min at 37°C. Dissociated cells were cultured in DMEM containing 10% FBS and 50 mg/ml gentamicin at 37°C the culture media was changed every 3 to 4 days. Astrocytes were passaged for maximum of three times and exposed at 80-90% confluency. For analysis of culture purity, cells were stained with microglia marker Iba-1 (ionized calcium binding adapted molecule 1), pericytes markers NG-2 and PDGF-Rβ (platelet-derived growth factor β receptor), endothelial cells markers Isolectin-B4 and tyrosine-protein kinase receptor TIE-2 and astrocytic marker GFAP. 95% of the cells were GFAP-positive and negative for the other markers, therefore considered astrocytic.

Hypoxic, glucose deprivation and drug exposure experiments

Primary astrocytes were incubated in 5% CO_2-containing atmospheres at 37ºC under normoxic (room air), hypoxic (1% O_2) and near-anoxic (0.2% O_2) conditions in a humidified glove box incubator (Invivo$_2$ 200; Ruskinn Technologies) for 16 or 36 hours. Glucose deprivation experiments were carried out with glucose-free media. mTOR inhibitor rapamycin or PI3K/mTOR inhibitor NVP (NVP-BEZ235 hydrochloride) were used at a concentration of 10 nM and 250 nM, respectively to inhibit 4E-BP1 phosphorylation.

Transfection

Astrocytes were plated and allowed to reach 80-90% confluency (except where differently specified). Cells were transfected with 100 nM siRNA using Oligofectamine™ Reagent according to manufacturer's instructions. Briefly, Oligofectamine was diluted in Opti-MEM® and incubated 5 min at room temperature (RT). In parallel, ON_TARGETplus SMARTpool siRNA or siCONTROL Non-Targeting scrambled RNA were combined with Opti-MEM®, added to the Oligofectamine/Opti-MEM mixture and incubated 20 min at RT. Cells were washed once with PBS, medium was replaced with DMEM Glutamax II and the siRNA duplexes were added, mixed gently and incubated for 5.5 hours at 37°C/5% CO_2 before the media was replaced with media containing serum and antibiotics. The next day cells were exposed to oxygen and/or glucose deprivation and/or drug for 16 hours and then analyzed.

Immunoblotting

Cell lysates were generated using cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton, 1% NP-40) supplemented with protease inhibitor cocktail, sodium orthovanadate (1 mM), dithiothreitol (0.5 mM) and phenylmethansulfonyl fluoride (0.5 mM), incubated 10 min on ice and then cleared by centrifugation for 10 min at 16,000 g at 4°C. Protein concentrations were quantified with the Bio-Rad protein assay kit. 30 µg lysates were generated using Laemli buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 25% glycerol, 0.02% bromophenol blue, 25% 2-mercaptoethanol), then resolved on polyacrylamide gel with SDS running buffer (192 mM glycine, 25 mM Tris, 0.1% SDS) and transferred to nitrocellulose membrane. The membrane was blocked for 1 h at RT with 5% dry milk in Tris-buffered saline containing 0.1% Tween-20, probed with primary antibodies overnight at 4°C. Immobilized primary antibodies were visualized using HRP-conjugated secondary antibodies. Image analysis was done using ImageJ-image processing and analysis software (National Institute of Health), and normalized to β-actin or total protein levels.

BrdU incorporation and detection *in vitro*

Cell proliferation ELISA BrdU kit was used following manufacturer instructions. Briefly, 40 to 50% confluent cells were transfected and the following day exposed to BrdU. Subsequently, cells were fixed for 30 minutes, then incubated 1.5 hours at 37°C with a BrdU antibody conjugated to peroxidase (anti-BrdU-POD). Following a PBS wash POD substrate was added and optical density read

at 405 nm with reference at 520 nm. The measured absorbance is proportional to the cells in S-phase.

Cap-affinity assay

Astrocytes were lysed in freeze-thaw lysis buffer (FTLB; 50 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10 mM Tetrasodium pyrophosphate decahydrate) containing protease inhibitor cocktail, 1 mM sodium orthovanadate, 0.5 mM phenylmethansulfonyl fluoride. Cells were snap-frozen in liquid nitrogen and thawn three times and then centrifuged for 10 min at 16,000 g at 4°C. 170 µg proteins were incubated with 60 µl of m^7GTP-sepharose while mixing for 2 hours at 4°C. The sepharose was then washed twice and the captured proteins eluted in elution buffer (25 mM Tris-HCl pH 7.5, 150mM KCl) containing 100 µM 7-methylguanosine 5'-triphosphate for 1 hour at 4°C. Eluted proteins were then prepared for immunoblotting.

Protein synthesis rate analysis: metabolic labeling with [^{35}S]-methionine and cysteine

Cells were labeled for 30 minutes with 10 µCi/ml of [^{35}S]-methionine/cysteine in methionine and cysteine-free media supplemented with 0.1% serum, washed three times with cold PBS and protein precipitated with cold 10% trichloroacetic acid twice for 30 minutes on ice. Precipitated proteins were solubilized with 0.1% SDS, 0.1M NaOH for 30 minutes at 37°C shaking occasionally, neutralized with 1 M acetic acid, then counted in a liquid scintillation counter (Packard, Tri-Carb1600 RT).

Statistical analysis

All data were analyzed using Prism 5 software. After having confirmed normal distribution of respective data populations the comparison between two groups was performed using Two-way ANOVA followed by Bonferroni post-hoc test. $P < 0.05$ was considered statistically significant

RESULTS

Astrocytic 4E-BP1 and S6K1 have different sensitivity to rapamycin

We investigated changes in 4E-BP1 and S6K1 phosphorylation by immunoblot under normal and stress conditions (Fig. 1A). Under normoxic conditions (Nx, room air) 4E-BP1 was hyper-phosphorylated (γ and β bands) and hypoxic (Hx, 1% O_2) or near-anoxic (Ax, 0.2% O_2) exposure (16 hours each) did not alter this state. In contrast ischemic conditions (near-anoxia and glucose deprivation) yielded considerable hypo-phosphorylation of 4E-BP1 (α band). It is well known that in cancer cells 4E-BP1 regulation mainly depends on mTORC1 activity (7, 21, 22). To test the effect of this kinase on 4E-BP1 phosphorylation, we treated cells with 10 nM rapamycin, a specific mTORC1 inhibitor. The 10 nM dose was chosen based on literature findings (23) and pilot experiments revealed this concentration as non-toxic (data not shown). Surprisingly, rapamycin treatment only partially dephosphorylated 4E-BP1 (γ, β and α bands) independent of oxygen and glucose exposure (Fig. 1A). In contrast phosphorylation of S6K1, another downstream target of mTORC1, was moderately inhibited during sole oxygen deprivation, yet completely abrogated during ischemia (Ax-G; Fig. 1A). As expected, rapamycin dephosphorylated S6K1 in all conditions, indicating differential sensitivity of 4E-BP1 and S6K1 to rapamycin driven mTORC1 inhibition. Since AKT has been shown to directly phosphorylate 4E-BP1 (24, 25), we hypothesized a role for this kinase in such regulation. Particularly S6K1 dephosphorylation by prolonged rapamycin application is known to activate a feedback loop involving AKT (26). Under our conditions, oxygen deprivation did not affect AKT phosphorylation, while glucose withdrawal slightly decreased it. Interestingly, rapamycin triggered a pronounced elevation of AKT

phosphorylation in all conditions except ischemia (Fig. 1A). These results suggest that in primary astrocytes S6K1 phosphorylation is inhibited with far greater sensitivity to oxygen reduction or rapamycin exposure, while only severe (ischemic) conditions inactivate mTORC1- and AKT-relayed signaling onto 4E-BP1.

To determine if varying degrees of 4E-BP1 phosphorylation impact overall protein synthesis in astrocytes, we performed protein synthesis rate (PSR) assays (Fig. 1B). Compared to the respective normoxic (Nx) reference, overall PSR dropped as a function of oxygen deprivation (Nx > Hx > Ax). Unexpectedly, simultaneous glucose withdrawal or rapamycin application had no additional effect (Fig. 1B), hence maximal activation (hypo-phosphorylation) of 4E-BP1 in Ax-G condition does not correlate with a major reduction in the global protein synthesis of astrocytes. We next assessed changes induced by oxygen and glucose deprivation on the activity of the binding protein using in vitro cap-capture assay with a specific antibody that detects non-phosphorylated (Thr46) in 4E-BP1 (Fig. 1C). This site initiates the hierarchical phosphorylation cascade that results in 4E-BP1 release from eIF4E (22). For clear discrimination of the interaction equilibria of hypo-phosphorylated 4E-BP1 with eIF4E we compared Nx versus Ax conditions, since hypo-phosphorylation of 4E-BP1 in primary astrocytes occurs only during severe Ax-G stress (Fig. 1A). This time astrocytes were challenged for 16 and 36 hours periods to see if i) 16 hour of ischemia (Ax-G) corresponds to an optimal degree of 4E-BP1 activation, and ii) extended periods of severe injury could augment 4E-BP1 hypo-phosphorylation. 36h oxygen deprivation alone did not affect 4E-BP1 phosphorylation (data not shown) and only marginally strengthened the association of eIF4E with 4E-BP1

(Fig. 1C; 36h Nx+G versus Ax+G). In contrast, 16 and 36h glucose deprivation clearly increased capture of eIF4E by non-phosphorylated (active) 4E-BP1. It should be noted that despite increased hypo-phosphorylated 4E-BP1 at 36h Ax-G, the ability of 4E-BP1 to bind eIF4E was not affected. The heightened capacity of dephosphorylated 4E-BP1 to interact with eIF4E during ischemia was also reflected by reduced interaction between eIF4G and eIF4E (Fig. 1B) for both 16 and 36 hours exposure.

4E-BP1 knockdown reduces both protein synthesis and cell cycle rate in astrocytes

Next we knocked down 4E-BP1 using siRNA to investigate the effect of loss-of-function of the hyper- (in Nx±G) or hypo-phosphorylated (in Ax-G) factor on overall PSR and cell cycle regulation (Fig. 2). 80% knockdown of 4E-BP1 by siRNA (Fig. 2A) did not affect cell viability in any condition (data not shown). Interestingly, 4E-BP1 silencing reduced the rate of protein synthesis to 70% of the control (Scrm) during normoxic conditions independently of glucose exposure but unexpectedly did not alter mRNA translation during near-anoxic exposure (Fig. 2B). These data led us to hypothesize that hyper-phosphorylated 4E-BP1 might act as potential stimulator of protein synthesis in oxygenated astrocytes, a notion further supported by measuring cell proliferation using BrdU incorporation (Fig. 2C). Although oxygen and glucose deprivation did not produce measurable differences in proliferative activities, 4E-BP1 knockdown induced a trend to decreased cell cycle progression in all conditions and significantly inhibited BrdU incorporation down to 80% of control values during normoxia (Fig. 2C). We also attempted to reverse 4E-BP1-knockdown mediated

inhibitory effects by overexpressing a constitutively active (dephosphorylated) mutant form of 4E-BP1 (Thr-37–Glu/Thr-46–Glu 4E-BP1). However, overexpression of the double mutant 4E-BP1 did not affect protein synthesis or BrdU incorporation rates (data not shown). We infer that the hyper-phosphorylated 4E-BP1 species plays a major and positive role in mRNA translation and cell cycle regulation during non-stress conditions in primary astrocytes.

4E-BP1 knockdown suppresses S6K1 but not AKT, eIF2α or eIF4E

We investigated the effect of 4E-BP1 knockdown on players of both cap- and TOP-dependent translation (Fig. 3), which ultimately regulate cell proliferation. Since normoxia and near-anoxia minus glucose were sufficient to distinguish between actions of hyper- versus hypo-phosphorylated 4E-BP1, subsequent experiments were performed under these two conditions. Surprisingly, 4E-BP1 knockdown significantly decreased total levels of S6K1 during normoxia and, although not statistically significant, caused a similar reduction during ischemic conditions (Fig. 3A&B). While oxygen deprivation had no effect on total levels of S6K1, the phosphorylation of this mTORC1 effector was inhibited during ischemia. 4E-BP1 knockdown seemed to reduce phosphorylated S6K1 only as a consequence of decreased total S6K1 levels (Fig. 3A&C). Total AKT did not change in any of the conditions (Fig. 3A), while in contrast Ax-G significantly decreased AKT phosphorylation. Notably, 4E-BP1 knockdown had no effect on AKT (Fig. 3A&D). Initiation factor eIF4E and total eIF2α were also not affected either by oxygen deprivation or 4E-BP1 manipulation (Fig. 3A), although phosphorylated eIF2α increased during oxygen withdrawal (Fig. 3A&E). These

results suggest that in primary astrocytes 4E-BP1 promotes expression and activity of the TOP-protein synthesis regulator S6K1 but not of any of the investigated cap-translation related proteins.

S6K1 knockdown mimics the effect of 4E-BP1 gene silencing on overall protein synthesis but not cell proliferation.

As 4E-BP1 knockdown decreased S6K1 levels (Fig. 3A&B), we hypothesized that phosphorylated 4E-BP1 promotes PSR and cell growth via S6K1 regulation. To investigate the consequence of reduced S6K1-mediated TOP-dependent translation on overall protein synthesis and cell proliferation, we used siRNA to silence transcription of S6K1 alone and in combination with 4E-BP1 (Fig. 4A). While si4E-BP1 again weakened S6K1 levels, knockdown of S6K1 did not impact abundance of 4E-BP1 protein. Interestingly, S6K1 knockdown reduced protein synthesis rates almost as effectively as 4E-BP1 depletion under Nx+G condition, although not statistically significant. However, genetic manipulation of S6K1 neither affected the rate of mRNA translation during glucose withdrawal nor oxygen deprivation (Fig. 4B). These results indicate that S6K1 may regulate the decrease of PSR caused by 4E-BP1 knockdown only during normal conditions. Unexpectedly, DNA replication was not affected by gene silencing of S6K1 in any of the conditions (Fig. 4C), while double knockdown of S6K1 and 4E-BP1 (si4E-BP1/S6K1) reduced DNA replication to the same extent as 4E-BP1 genetic manipulation alone (si4E-BP1). Thus loss of S6K1 does not alter BrdU incorporation and therefore it is not implicated as a trigger for astrocyte cell cycle regulation.

The inhibitor NVP blunts the effect of 4E-BP1 gene silencing

To further assess the contribution of hyper-phosphorylated 4E-BP1 to mRNA translation and cell proliferation in oxygenated astrocytes, we fully dephosphorylated 4E-BP1 by treating normoxic astrocytes with NVP-BEZ235 (NVP), a potent ATP-competitive PI3K and mTOR inhibitor (27). NVP treatment inhibited 4E-BP1 phosphorylation in a dose-dependent manner, with non-toxic 250 nM causing its complete dephosphorylation (α band only) (Fig. 5A). Next we additionally used siRNA to knockdown 4E-BP1 and performed PSR and BrdU incorporation assay. Surprisingly, 250 nM NVP reduced protein synthesis to 50% of the control (Scrm Nx+G) independently of glucose exposure (Fig. 5B). Furthermore, drug treatment completely blunted the effect of 4E-BP1 knockdown on PSR during normoxia. In complete contrast, applying NVP to cells subjected to anoxia did not yield any attenuation in global protein synthesis activity (Fig. 5C). NVP treatment also caused a 50% decrease in BrdU incorporation during both normoxic and near-anoxic conditions independently of glucose deprivation and, here again, completely blunted the effect of 4E-BP1 knockdown (Fig. 5D). Taken together these data underline the hypothesis that hyper-phosphorylated 4E-BP1 rather than the dephosphorylated protein stimulates overall protein synthesis and cell cycle progression in primary astrocytes during non-stress conditions.

DISCUSSION

4E-BP1 has been shown to play a pivotal role in the regulation of protein synthesis and cell survival in cancer cells during disease progression. However

the physiological function of this important regulator in primary cells still needs to be elucidated. This study shows that during stress conditions astrocytic 4E-BP1 inhibits cap-dependent translation when hypo-phosphorylated similar to the mechanisms observed in cancer cells. However, we now provide novel evidence that phosphorylated 4E-BP1, rather than being an inactive mTORC1 pathway component, actively stimulates cell proliferation and overall protein synthesis under normal conditions.

Differential sensitivity of 4E-BP1 and S6K1 to rapamycin-induced mTORC1 inhibition in cancer cells as well as in myoblasts was recently described (26, 28, 29). Similarly, although ischemia completely dephosphorylated 4E-BP1 and S6K1 in primary astrocytes, anoxia or rapamycin treatment inhibited S6K1 but not 4E-BP1 phosphorylation. This observation suggested that other rapamycin-refractory kinases also contribute to 4E-BP1 regulation. In our study the kinase AKT was activated by prolonged rapamycin treatment, similar to data from others (26) showing that S6K1 dephosphorylation by rapamycin results in the loss of a negative feedback loop which eventually resulted in the activation of AKT in bladder cancer cells. Other groups have shown in human embryonic kidney cells as well as in cancer cells that AKT signaling directly mediates 4E-BP1 phosphorylation (24, 25). We speculate that in primary astrocytes S6K1 dephosphorylation may induce S6K1-dependent AKT activation and thereby sustain 4E-BP1 phosphorylation.

During severe stress conditions (i.e. ischemia) dephosphorylated (active) 4E-BP1 inhibited cap-dependent translation through interaction with eIF4E in analogy to published data obtained from both, transformed (30) and primary cells (13). Combining glucose with oxygen deprivation also prohibited the

partnering between eIF4E and eIF4G within the eIF4F cap-binding, which mirrors observations in anoxic HeLa cells (31). Nevertheless, ischemic inhibition of astrocytic cap-dependent translation through dephosphorylated 4E-BP1 did not further intensify inhibition of overall protein synthesis compared to anoxia (where hyper-phosphorylated 4E-BP1 prevails). These findings are in agreement with a previous study on isolated rat hepatocytes demonstrating the inadequacy of rapamycin-dependent 4E-BP1 dephosphorylation to cause a decrease in global protein synthesis (13). This contrasts with the observation that 4E-BP1 in immortalized breast epithelial cells partially regulates global translation during hypoxia (32). Thus, tumor cells may be more susceptible than non-transformed cells to the inhibitory role of 4E-BP1 due to their high PSR. In our more slowly dividing astrocytes we failed to obtain any evidence implicating dephosphorylated 4E-BP1 in the regulation of global translation during severe stress, i.e. under conditions when the factor scavenges eIF4E from the cap-translation promoting eIF4F complex.

While its canonical activities were restricted to extreme challenges, 4E-BP1 surprisingly stimulated protein synthesis under normal circumstances when oxygen and substrates were abundantly available. Furthermore, knockdown of the allegedly inactive, i.e. hyper-phosphorylated, species reduced overall PSR and decreased cell proliferation under normoxia but had no effect during near-anoxia. These findings, support the hypothesis that hyper-phosphorylated 4E-BP1 promotes protein synthesis and cell cycle progression during non-stress conditions. Indeed regulation of cell cycle by 4E-BP1 could be an indirect consequence of altered protein synthesis or vice versa. In good agreement recent evidence showed phosphorylated 4E-BP1 levels to increase during mitosis

where the factor is believed to play a role in cell division in both primary retinal cells (33) and some transformed cell lines (15, 16). Moreover, a recent study correlated 4E-BP1 phosphorylation levels with glioblastoma grade (10). Phosphorylated 4E-BP1 could influence both cell cycle and protein synthesis by regulating different proteins including S6K1, AKT, eIF2α and eIF4E, key factors in the control of mRNA translation and cell proliferation via AKT/mTOR pathway (34). We demonstrated that astrocytes subjected to oxygen and glucose deprivation inactivate S6K1 and AKT in parallel with the induction of phosphorylated eIF2α. Interestingly, 4E-BP1 knockdown diminished total and phosphorylated S6K1 levels, while genetic manipulation of S6K1 had no effect at all on 4E-BP1. S6K1's role in regulating TOP-dependent translation suggests that its down regulation may additionally contribute to decreased global protein synthesis when 4E-BP1 is depleted. Indeed, it was already observed that S6K1 is involved in coordinating the assembly of the translation pre-initiation complex (35). Astrocytic S6K1 knockdown was as effective as the 4E-BP1 loss-of-function in decreasing PSR in normoxic/glucose-proficient cells, but not in normoxic/glucose-deficient cells, suggesting modulation of protein synthesis in a glucose and oxygen dependent manner. Hence, in oxygenated cells, phosphorylated 4E-BP1 might stimulate protein synthesis through S6K1. Deletion of S6K1 in published studies impaired proliferation of cultured hepatocytes and human U2OS osteosarcoma cells (36, 37) but not skeletal muscle (38) or murine primary astrocytes (39). In agreement with the above studies of non-cancer models, our data also showed that S6K1 knockdown did not impact astrocyte cell cycle indicating that loss of S6K1 may contribute to

reduced protein synthesis but not decreased cell proliferation after 4E-BP1 depletion.

Importantly, the present study emphasizes that deletion of hyper-phoshorylated 4E-BP1 in oxygenated glia decreased PSR and cell proliferation. So far the impact of phosphorylated forms of 4E-BP1 remains largely uninvestigated. Although some recent studies demonstrated involvement of phosphorylated 4E-BP1 in regulating mitotic division and cell cycle progression (15, 16, 33), to our knowledge it has never been shown that hyper-phosphorylated 4E-BP1 can act as stimulator of protein synthesis. Under normal conditions 4E-BP1 triggered mRNA translation and cell proliferation whereas inhibition of the PI3K/AKT/mTOR pathway via NVP abrogated 4E-BP1 phosphorylation and completely blunted the effect of 4E-BP1 knockdown on global translation regulation. It is worth noticing in this context, that maximal interference with PI3K/AKT/mTOR signaling (complete dephosphorylation of 4E-BP1) by NVP in normoxic/glucose-proficient astrocytes resulted in ~50% reduction of PSR, whereas deficiency of phosphorylated 4E-BP1 caused protein synthesis to drop by ~30%. By these measures, only half of the astrocytic capacity to generate polypeptides is controlled by the PI3K/AKT/mTOR pathway. Thus phosphorylated 4E-BP1 controls roughly 60% of the bulk protein synthesis pathway. In reality, the binding proteins' control index is likely to even exceed 60%, given the incomplete efficacy of the knockdown. Thus 4E-BP1 dephosphorylation inhibits cap-dependent translation as expected whereas hyper-phosphorylation actively stimulates overall protein synthesis. These results clearly indicate that hyper-phosphorylated 4E-BP1 is not inactive as is commonly thought but has a rather widespread impact on stimulating the

synthesis of bulk protein and DNA during normal growth conditions. However, considering NVP treatment had a stronger effect than 4E-BP1 knockdown alone, other players of the PI3K/AKT/mTOR pathway must also be involved.

At least for primary astrocytes, 4E-BP1 exists as Janus-faced molecule, where its hyper- and hypo-phosphorylated forms exert opposing effects on protein synthesis and cell cycle progression. This study provides novel information on mechanisms stimulating astrocytic proliferation that will help to identify new targets to suppress exaggerated growth in nonmalignant (e.g. astrogliosis) and malignant (e.g. astrocytoma) CNS pathologies. Clearly, more detailed examination of how (hyper)phosphorylated 4E-BP1 is able to trigger mRNA translation and cell proliferation in non-stressed backgrounds and in different cells is needed. The current prevailing doctrine that ascribes activity only to the hypo-/dephosphorylated form of 4E-BP1 clearly needs to be revisited.

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FIGURE LEGENDS

Fig. 1 Astrocytic 4E-BP1 and S6K1 have differential sensitivity to rapamycin

A) Western blot of total and phosphorylated S6K1, AKT and 4E-BP1 in astrocytes exposed to room air (Nx), 1% O_2 (Hx) and 0.2% O_2 (Ax), with (+) and without (-) glucose, with (+) and without (-) rapamycin treatment (10 nM) for 16 hours. β-actin was used as loading control. 4E-BP1 γ/β bands: hyper-phosphorylated protein; 4E-BP1 α band: hypo-phosphorylated protein; B) Protein synthesis rate was measured as percentage of incorporation of [^{35}S]-methionine and [^{35}S]-cysteine into cellular proteins. Following 16h incubation cells were exposed for 30 minutes to radiolabeled media, then proteins were precipitated with cold 10% trichloroacetic acid and incorporation of [^{35}S] was measured by counts per minute (CPM). Graph represents percentage CPM of total counts compared to Nx+G. Values are means ± SD (n=3). *p<0.05, **p<0.01, ****p<0.0001. C) *In vitro* assessment of eIF4F complex integrity employing a cap-affinity pull-down assay. Cells were exposed to Nx and Ax, +G and –G for 16 and 36 hours, protein extracts were subjected to pull-down assay with 7-Methyl GTP sepharose beads (7-Met GTP) before immunoblot analysis of eIF4E, eIF4G and non-phospho (Thr46) 4E-BP1. Whole cell extract (Input) was used as positive control for protein extraction, 7-Met GTP was used as negative control and β-actin to monitor pull down efficiency.

Fig. 2 4E-BP1 knockdown reduces both protein synthesis and cell cycle rate in astrocytes.

Astrocytes were transfected with 4E-BP1 siRNA (si4E-BP1) or non-targeting scrambled (Scrm) siRNA and then exposed to 16 hours of room air (Nx) or 0.2% O_2 (Ax), with (+G) or without glucose (-G). A) Knockdown efficiency was evaluated by Western blot of 4E-BP1. All samples were run on the same blot, however image was cropped due to sample redundancy. B) Protein synthesis rate measurement. Details as in Fig. 1. C) Cell proliferation was measured by BrdU incorporation. BrdU was incorporated for 16 hours, measured by optical density (OD) at 450 nm (reference of 620 nm) and plotted as percentage versus Scrm Nx+G. Values are means ± SD (n≥3). *p<0.05, **p<0.01, ***p<0.001.

Fig. 3 4E-BP1 knockdown suppresses S6K1 but not AKT, eIF2α or eIF4E

Astrocytes were transfected with 4E-BP1 siRNA (si4E-BP1) or non-targeting scrambled (Scrm) siRNA and then exposed to 16 hours room air (Nx) or 0.2% O_2 (Ax) without glucose (-G). A) Whole cell lysates were subjected to Western blotting with antibodies specific to total and phosphorylated (p) S6K1, AKT, eIF2α. All samples were run on the same blot, however image was cropped due to sample redundancy. Protein levels were quantified by densitometry using ImageJ software, graphs represent ratio of B) S6K1 to β-Act , C) p-S6K1 to total S6K1 , D) p-AKT to total AKT and E) p-eIF2α to total eIF2α . Values are means ± SD (n=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Fig. 4 S6K1 knockdown mimics the effect of 4E-BP1 gene silencing on overall protein synthesis but not cell proliferation

Astrocytes were transfected with S6K1 siRNA (siS6K1) or 4E-BP1 siRNA (si4E-BP1) or combination of both siRNAs (si4E-BP1/siS6K1) prior to exposure to 16 hours of room air (Nx) or 0.2% O_2 (Ax), with (+G) or without glucose (-G). Non-targeting scrambled (Scrm) siRNA was used as control. A) Western blot assessed efficiency of S6K1 and 4E-BP1 knockdown. B) Protein synthesis rate measurements. Details as in Fig. 1. C) Cell proliferation was measured by BrdU incorporation for 16 hours in the different conditions. Details as in Fig. 2. Values are means ± SD (n=3). *p<0.05, **p<0.01, ****p<0.0001.

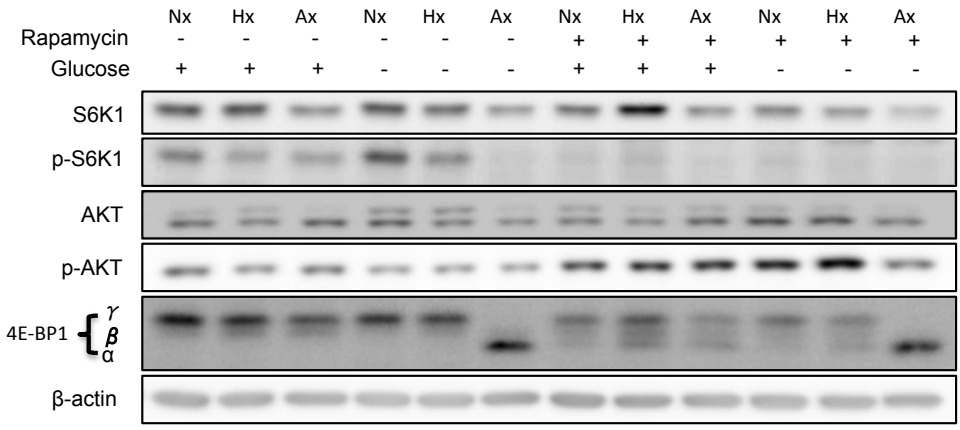
Fig. 5 The inhibitor NVP blunts the effect of 4E-BP1 gene silencing

A) Western blot of 4E-BP1 in astrocytes exposed to room air (Nx) or 0.2% O_2 (Ax), with increasing concentrations of NVP-BEZ235 hydrochloride (NVP), a potent ATP-competitive PI3K and mTOR inhibitor, for 16 hours. β-actin was used as loading control. All samples were run on the same blot, however image was cropped due to sample redundancy. B) Protein synthesis rate (PSR) of cells transfected with 4E-BP1 siRNA (si4E-BP1) or non-targeting scrambled (Scrm) RNA and subsequent exposure (16 hours) to room air (Nx) with (+G) or without glucose (-G), with or without 250 nM NVP. PSR measurements as in Fig. 1. C) PSR after 16 hours exposure to 0.2% O_2 (Ax) +G or –G, with or without 250 nM NVP. PSR measurements as in Fig. 1. Graphs in (B) and (C) show percentage of radiolabel incorporation compared to Scrm Nx+G (B) and Ax+G (C), respectively. D) Cell proliferation. 16 hours BrdU incorporation was measured as detailed in

Fig. 2. In all graphs values are means ± SD (n=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Fig. 1

A


Nx Hx Ax Nx Hx Ax Nx Hx Ax Nx Hx Ax
Rapamycin - - - - - - + + + + + +
Glucose + + + - - - + + + - - -
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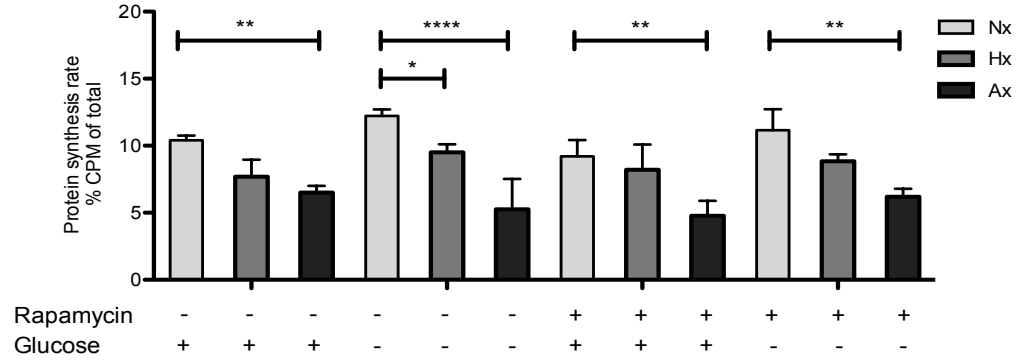
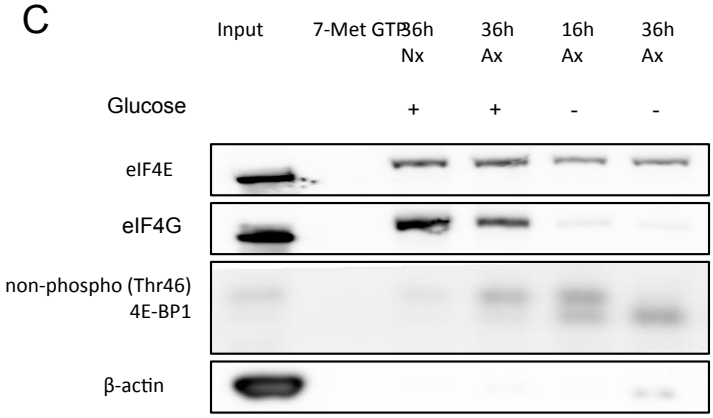
p-S6K1
AKT
p-AKT
4E-BP1
γ
β
α
β-actin

B


Protein synthesis rate
% CPM of total
20
15
10
5
0
**
****
*
**
**
Nx
Hx
Ax
Rapamycin - - - - - - + + + + + +
Glucose + + + - - - + + + - - -

C


Input
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36h Nx
36h Ax
16h Ax
36h Ax
Glucose + + - -
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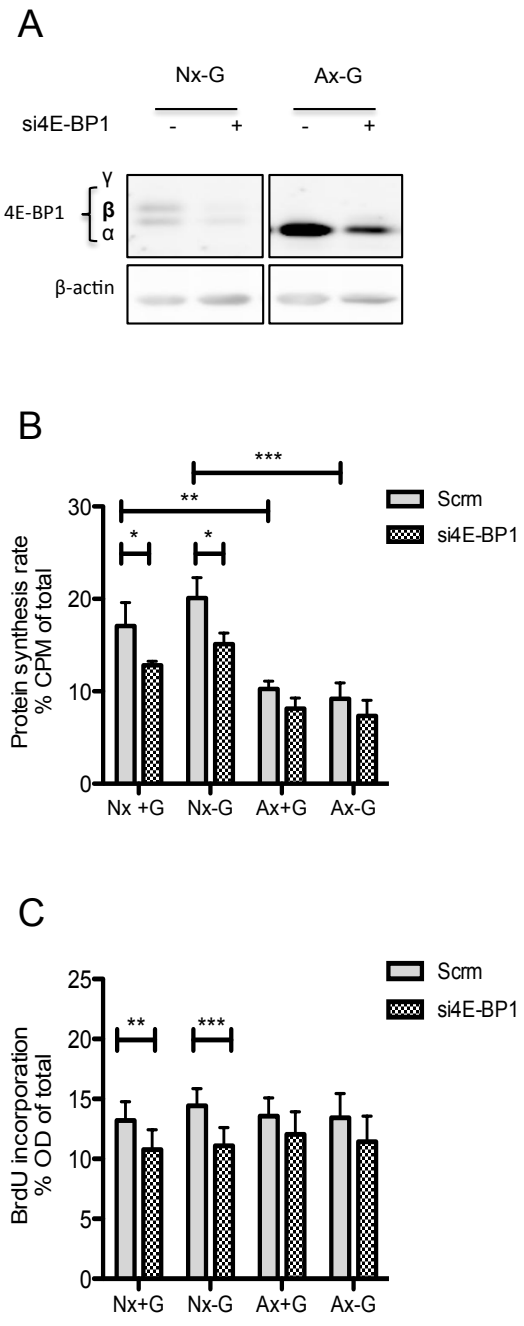
eIF4G
non-phospho (Thr46) 4E-BP1
β-actin

Fig. 2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4E-BP1
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β-actin
B
Protein synthesis rate
% CPM of total
Scrm
si4E-BP1
Nx +G
Nx-G
Ax+G
Ax-G
C
BrdU incorporation
% OD of total
Scrm
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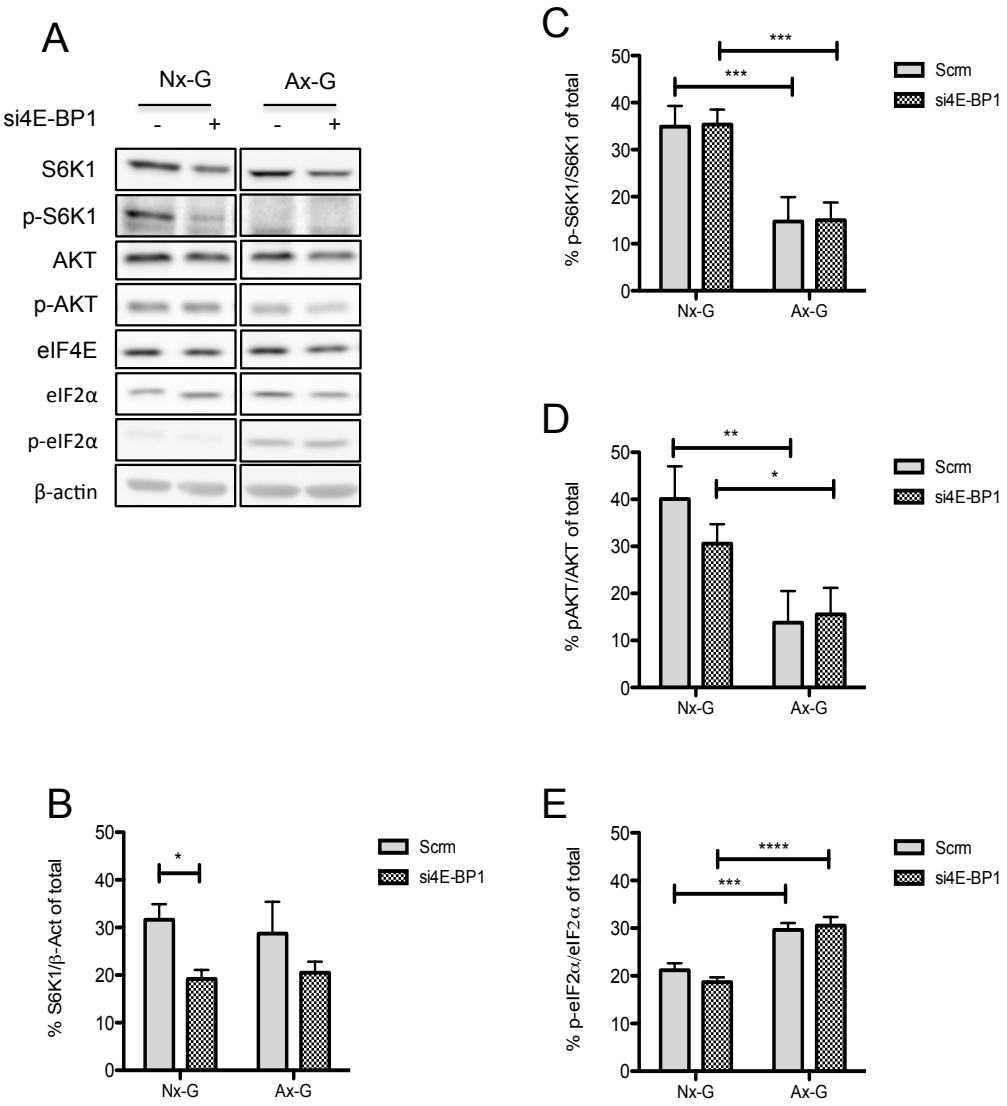
Nx+G
Nx-G
Ax+G
Ax-G

Fig. 3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p-eIF2α
β-actin
B
% S6K1/β-Act of total
Scrm
si4E-BP1
Nx-G
Ax-G
C
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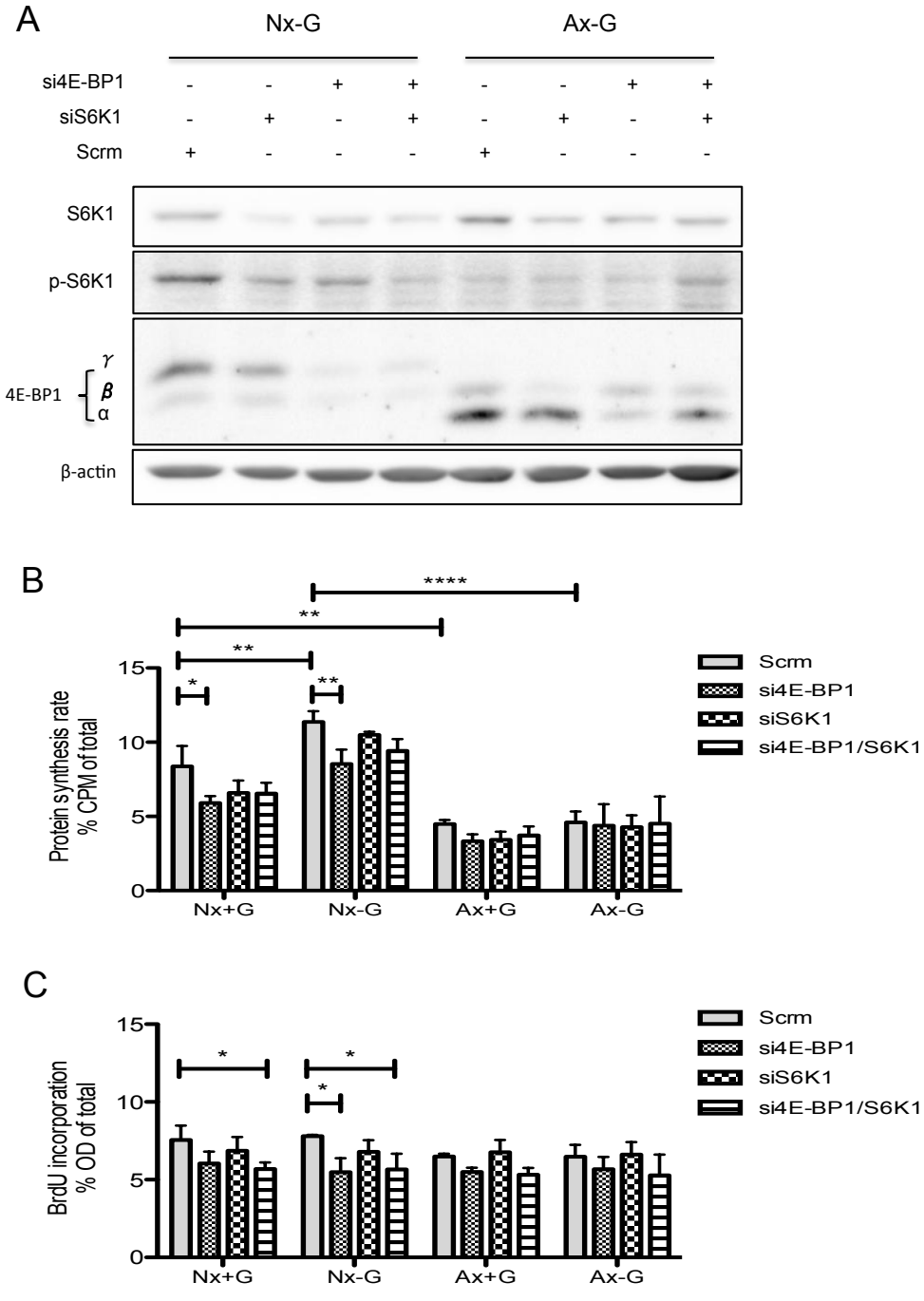
D
% pAKT/AKT of total
E
% p-eIF2α/eIF2α of total

Fig. 4


A
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Ax-G
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siS6K1
Scrm
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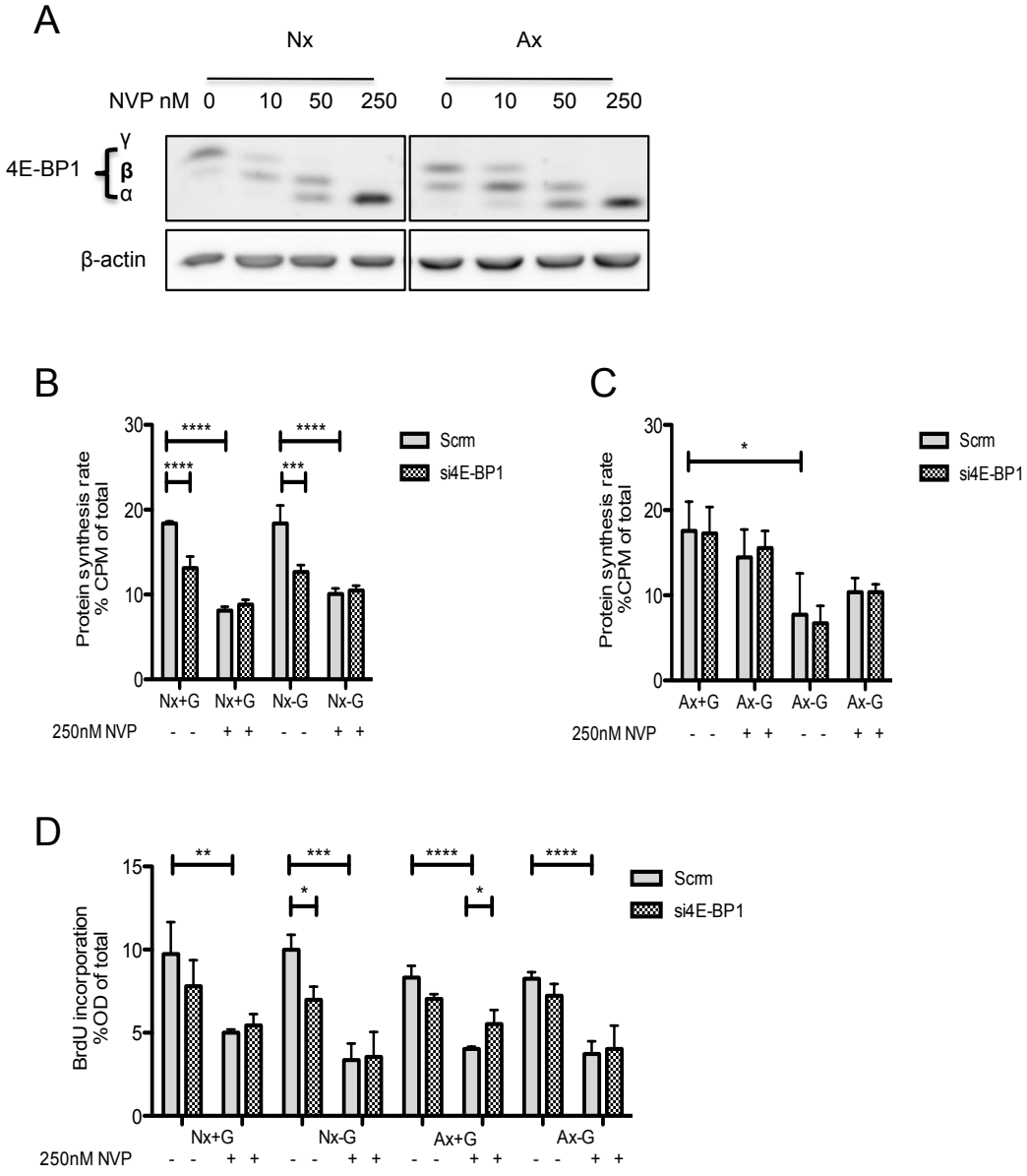
p-S6K1
4E-BP1
γ
β
α
β-actin
B
Protein synthesis rate
% CPM of total
Nx+G
Nx-G
Ax+G
Ax-G
Scrm
si4E-BP1
siS6K1
si4E-BP1/S6K1
C
BrdU incorporation
% OD of total
Nx+G
Nx-G
Ax+G
Ax-G
Scrm
si4E-BP1
siS6K1
si4E-BP1/S6K1

Fig.5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Protein synthesis rate
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250nM NVP - - + + - - + +
C
Protein synthesis rate
%CPM of total
Ax+G Ax-G Ax-G Ax-G
D
BrdU incorporation
%OD of total
Nx+G Nx-G Ax+G Ax-G

11 Curriculum vitae

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Oct. 2006 - May 2009 **Pharmaceutical Biotechnology Master's degree**
Università degli Studi di Perugia, Italy
Thesis supervised by Prof. Ursula Grohmann (Università degli Studi di Perugia, Italy) and Prof. Stéphanie Pochet (Université Libre de Bruxelles, Belgium)
Title of Thesis: *Toxicologic studies of passive smoke extract in culture of endothelial cells from human umbilical cord*.
Grade: 110/ 110 cum laude

Oct. 2003- Oct. 2006 **Biotechnology (Pharmaceutical) Bachelor's degree**
Università degli Studi di Perugia, Italy
Thesis supervised by Prof. Ursula Grohmann and Dr. Carmine Vacca
Title of Thesis*: Development of monoclonal anti- mouse IDO antibody in the rabbit*.
Grade: 106/110

Sept. 2002 - July 2003 **Secondary school diploma for foreign languages**.
Istituto Magistrale Statale ad Indirizzo Sperimentale Corrado Alvaro Palmi (RC), Italy
Diploma Maturità Magistrale Indirizzo Linguistico (Progetto Brocca)
Grade: 100/100

Presentations

2013 Poster presentation at the 9th Symposium of the Zurich Center for Integrative Human Physiology (ZHIP), Zürich.
Title: *4EBP1: a novel regulator of cycle progression in primary astrocytes?*

2012 Poster presentation at the Annual meeting of the Swiss Physiological Society, Fribourg.

Title: *Is 4E-Bp1 the hypoxia response regulator in differentially sensitive brain cells?*

2012 Poster presentation at the 8th Symposium of the Zurich Center for Integrative Human Physiology (ZHIP), Zürich.
Title: *Is 4E-Bp1 the hypoxia response regulator in differentially sensitive brain cells?*

2012 Poster presentation at the ZNZ Symposium, Zurich.
Title: *Is 4E-Bp1 the hypoxia response regulator in differentially sensitive brain cells?*

2011 Poster presentation at the 7th Symposium of the Zurich Center for Integrative Human Physiology (ZHIP), Zürich.
Title: *Does 4E-Bp1 regulate the hypoxic response in cells of different sensitivity?*

2011 Poster presentation at the «Imaging Life» USGEB Meeting 2011, Zurich.
Title: *Does mTOR signaling confer hypoxia resistance in brain cells?*

2011 Poster presentation at the 3rd meeting of COST Action: Hypoxia sensing, signaling and adaptation, Davos.
Title: *Does mTOR signaling confer hypoxia resistance in brain cells?*

2010 Poster presentation at the 6th Symposium of the Zürich Center for Integrative Human Physiology (ZHIP), Zürich.
Title*: Does mTOR signaling confer hypoxia resistance in brain cells?*

Technical skills

Cell culture: cell lines (HeLa, Hep3B, HUVEC, RBE4) freezing and expansion, rat primary astrocytes isolation and maintenance, gene manipulation (transfection and Lentiviral transduction), hybridoma technology

Molecular biology: protein purification, IP, Western blot, ELISA, EMSA, ICC, cytotoxicity assays (MTT, Trypan blue, Propidium Iodide), cell cycle assay (BrdU incorporation), nuclei acid extraction, PCR, metabolic labeling with [^{35}S]-methionine and cysteine

Languages

native Italian speaker
fluent English, Spanish and French
moderate German

References

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Dr. Thomas A. Gorr (thesis supervisor)
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12 Acknowledgements

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